A THESIS

entitled

STUDIES ON ABNORMAL ALBUMIN

by

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the requirements for the
degree of Doctor of Philosophy of
the University of Warwick,
in the Department of Chemistry and
Molecular Sciences

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To Mum,

with love

That is the essence of science:
ask an impertinent question,
and you are on the way
to the pertinent answer.

J. Bronowski

DECLARATION

The work described in this thesis was carried out in the Department of Chemistry and Molecular Sciences, University of Warwick, Coventry, England, during the period between November 1979 and September 1982. It is the original work of the author, except where specific acknowledgement is made or implied. This thesis has been submitted at the University of Warwick alone, in fulfilment of the requirements for the degree of Ph.D.

ACKNOWLEDGEMENTS

I should like to express my deep gratitude to my supervisor, Dr. D. W. Hutchinson, for his excellence in scientific acumen, supervision, concern and in maintaining a happy atmosphere in the laboratory. I am also grateful to the Chairman and staff of the Department of Chemistry and Molecular Sciences for permission to use their research facilities throughout this work. I am also indebted to the West Midlands Health Authority for financial support.

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Finally, my sincere thanks to my Mum and Teng Lew for their unwavering support.

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ABSTRACT

Albumin Mundey is a heat-stable monomeric, slow albumin variant. The mutation sites are located in CNBr fragments I (residues 1-87) and VI (residues 447-548). It does not have any additional peptide at the N- or C-terminal end as L-aspartic acid and L-leucine were detected as these respective residues. It is present in near equal quantity to normal albumin in serum and shares a common antigenicity with normal albumin. The isoelectric point of the defatted and native molecules are 5.7 and 5.0 respectively. The classification of its electrophoretic mobility using standard methods shows that it is similar to several Indian variants, but its dye binding properties are most similar to Albumin Kashmir. A study of its bilirubin binding capacity by the fluorescence-quenching method shows a slight impairment in binding.

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In a separate study, a rapid photoinduced isomerisation of bilirubin-IXα to an equilibrium mixture with bilirubin-IIIα and XIIIα was obtained in a buffer containing aqueous cationic detergent. Little isomerisation was detected under comparable conditions in anionic or neutral detergents.

PUBLICATIONS

1. The photoinduced isomerisation of bilirubin in cationic detergent solutions
Y. N. Au and D. W. Hutchinson (1980)
Biochem. J., 191, 657-659

2. ^{3}H-Labelled bilirubin and biliverdin
D. W. Hutchinson, N. M. Wilkes and H. Y. N. Au (1981) Journal of Labelled Compounds and Radiopharmaceuticals, Vol. XVIII, 1401-1404

CHAPTER I

NORMAL SERUM ALBUMIN

1.1 FUNCTIONS OF ALBUMIN

Albumin is a rugged multifunctional and versatile protein found as the main extracellular protein in blood plasma. Its physiological functions include binding of organic anions, inorganic cations, specific antibodies and the maintenance of the circulation by contributing 75-80% of the colloidal osmotic pressure of plasma[20]. Its life-saving properties are in the binding and detoxification of bilirubin, binding of toxins and is the panacea for circulatory collapse or metabolic depletion. Clinicians use the measurement of albumin as an indicator of vascular integrity, nutritional status and functional reserve of the liver. Minor functions are in the transport of thyroid and steroid hormones, and as a nutritive reservoir. These physiological functions of albumin do not depend on specific interactions but on the broad non-specific physiochemical character of the protein[23].

Protein chemists use albumin as a model for almost every conceivable chemical reaction and physiochemical measurement in the investigation of proteins. As it is one of the rare proteins without a carbohydrate prosthetic group, it is used as a standard reference in protein assay.

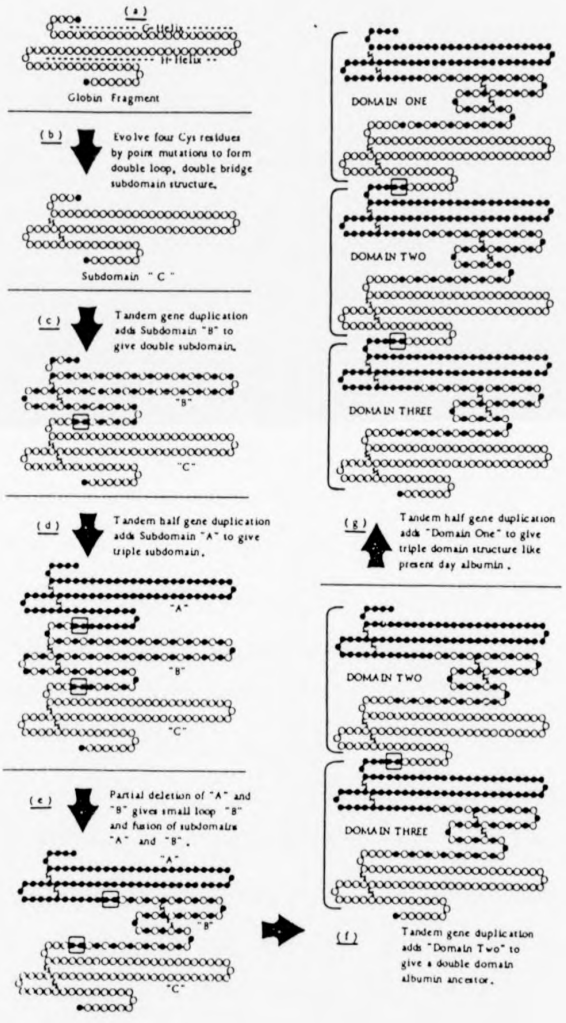

Fig. 1.1 The evolution of albumin according to Brown[47].

1.2 EVOLUTION OF ALBUMINS

Unlike the albumins found inconsistently in fish and amphibians, the albumins of birds and mammals are similar to human serum albumin (HSA) in solubility, charge, size and amino-acid composition[57]. Albumin-like molecules with mobilites ranging from 0.65 to 1.21 that of HSA are found in the four major reptilian orders, the snakes, crocodiles, lizards and turtles[175]. Inter-species immunochemical comparisons show that albumin has continued to evolve through non-functional (neutral) amino acid changes[176].

The evolutionary pathway of albumin from a primordial albumin as proposed by Brown[47] is shown in Fig. 1.1. Here a single double loop (such as loop 9) evolved into a triplet domain by a process of tandem gene duplication, half-duplication and deletion of approximately 40 residues from the middle loop. The primitive albumin in its equally primitive plasma may have specialised in binding of a small but specific range of compounds, but evolutionary conglomeration has resulted in a functionally-versatile protein with the capability to bind a wider range of compounds.

The age of the first duplication is estimated to be about one billion years and the last about 700 million years[46].

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Brown[47].

```
                5                  10                  15
  1  Asp-Ala-His-Lys-Ser-Glu-Val-Ala-His-Arg-Phe-Lys-Asp-Leu-Gly-
 16  Glu-Glu-Asn-Phe-Lys-Ala-Leu-Val-Leu-Ile-Ala-Phe-Ala-Gln-Tyr-
 31  Leu-Gln-Gln-Cys-Pro-Phe-Glu-Asp-His-Val-Lys-Leu-Val-Asn-Glu-
 46  Val-Thr-Glu-Phe-Ala-Lys-Thr-Cys-Val-Ala-Asp-Glu-Ser-Ala-Glu-
 61  Asn-Cys-Asp-Lys-Ser-Leu-His-Thr-Leu-Phe-Gly-Asp-Lys-Leu-Cys-
 76  Thr-Val-Ala-Thr-Leu-Arg-Glu-Thr-Tyr-Gly-Glu-Met-Ala-Asp-Cys-
 91  Cys-Ala-Lys-Glu-Gln-Pro-Glu-Arg-Asn-Glu-Cys-Phe-Leu-Gln-His-
106  Lys-Asp-Asp-Asn-Pro-Asn-Leu-Pro-Arg-Leu-Val-Arg-Pro-Glu-Val-
121  Asp-Val-Met-Cys-Thr-Ala-Phe-His-Asp-Asn-Glu-Glu-Thr-Phe-Leu-
136  Lys-Lys-Tyr-Leu-Tyr-Glu-Ile-Ala-Arg-Arg-His-Pro-Tyr-Phe-Tyr-
151  Ala-Pro-Glu-Leu-Leu-Phe-Phe-Ala-Lys-Arg-Tyr-Lys-Ala-Ala-Phe-
166  Thr-Glu-Cys-Cys-Glu-Ala-Ala-Asp-Lys-Ala-Ala-Cys-Leu-Leu-Pro-
181  Lys-Leu-Asp-Glu-Leu-Arg-Asp-Glu-Gly-Lys-Ala-Ser-Ser-Ala-Lys-
196  Gln-Arg-Leu-Lys-Cys-Ala-Ser-Leu-Gln-Lys-Phe-Gly-Glu-Arg-Ala-
211  Phe-Lys-Ala-Trp-Ala-Val-Ala-Arg-Leu-Ser-Gln-Arg-Phe-Pro-Lys-
226  Ala-Glu-Phe-Ala-Glu-Val-Ser-Lys-Leu-Val-Thr-Asp-Leu-Thr-Lys-
241  Val-His-Thr-Glu-Cys-Cys-His-Gly-Asp-Leu-Leu-Glu-Cys-Ala-Asp-
256  Asp-Arg-Ala-Asp-Leu-Ala-Lys-Tyr-Ile-Cys-Glu-Asn-Gln-Asp-Ser-
271  Ile-Ser-Ser-Lys-Leu-Lys-Glu-Cys-Cys-Glu-Lys-Pro-Leu-Leu-Glu-
286  Lys-Ser-His-Cys-Ile-Ala-Glu-Val-Glu-Asn-Asp-Glu-Met-Pro-Ala-
301  Asp-Leu-Pro-Ser-Leu-Ala-Ala-Asp-Phe-Val-Glu-Ser-Lys-Asp-Val-
316  Cys-Lys-Asn-Tyr-Ala-Glu-Ala-Lys-Asp-Val-Phe-Leu-Gly-Met-Phe-
331  Leu-Tyr-Glu-Tyr-Ala-Arg-Arg-His-Pro-Asp-Tyr-Ser-Val-Val-Leu-
346  Leu-Leu-Arg-Leu-Ala-Lys-Thr-Tyr-Glu-Thr-Thr-Leu-Glu-Lys-Cys-
361  Cys-Ala-Ala-His-Asp-Pro-Tyr-Glu-Cys-Ala-Ala-Lys-Val-Phe-Asp-
376  Glu-Phe-Lys-Pro-Leu-Val-Glu-Glu-Pro-Gln-Asn-Leu-Ile-Lys-Gln-
391  Asn-Cys-Glu-Leu-Phe-Glu-Gln-Leu-Gly-Glu-Tyr-Lys-Phe-Gln-Asn-
406  Ala-Leu-Leu-Val-Arg-Tyr-Thr-Lys-Lys-Val-Pro-Gln-Val-Ser-Thr-
421  Pro-Thr-Leu-Val-Glu-Val-Ser-Arg-Asn-Leu-Gly-Lys-Val-Gly-Ser-
436  Lys-Cys-Cys-Lys-His-Pro-Glu-Ala-Lys-Arg-Met-Pro-Cys-Ala-Glu-
451  Asp-Tyr-Leu-Ser-Val-Val-Leu-Asn-Gln-Leu-Cys-Val-Leu-Glu-His-
466  Lys-Thr-Pro-Val-Ser-Asp-Arg-Val-Thr-Lys-Cys-Cys-Thr-Glu-Ser-
481  Leu-Val-Asn-Arg-Arg-Pro-Cys-Phe-Ser-Ala-Leu-Glu-Val-Asp-Glu-
496  Thr-Tyr-Val-Pro-Lys-Glu-Phe-Asn-Ala-Glu-Thr-Phe-Thr-Phe-His-
511  Ala-Asp-Ile-Cys-Thr-Leu-Ser-Glu-Lys-Glu-Arg-Gln-Ile-Lys-Lys-
526  Gln-Thr-Ala-Leu-Val-Glu-Leu-Val-Lys-His-Lys-Pro-Lys-Ala-Thr-
541  Lys-Glu-Gln-Leu-Lys-Ala-Val-Met-Asp-Asp-Phe-Ala-Ala-Phe-Val-
556  Glu-Lys-Cys-Cys-Lys-Ala-Asp-Asp-Lys-Glu-Thr-Cys-Phe-Ala-Glu-
571  Glu-Gly-Lys-Lys-Leu-Val-Ala-Ala-Ser-Gln-Ala-Ala-Leu-Gly-Leu
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Fig. 1.2 The complete amino acid sequence of human serum albumin[184] (cysteine-34: free SH group)

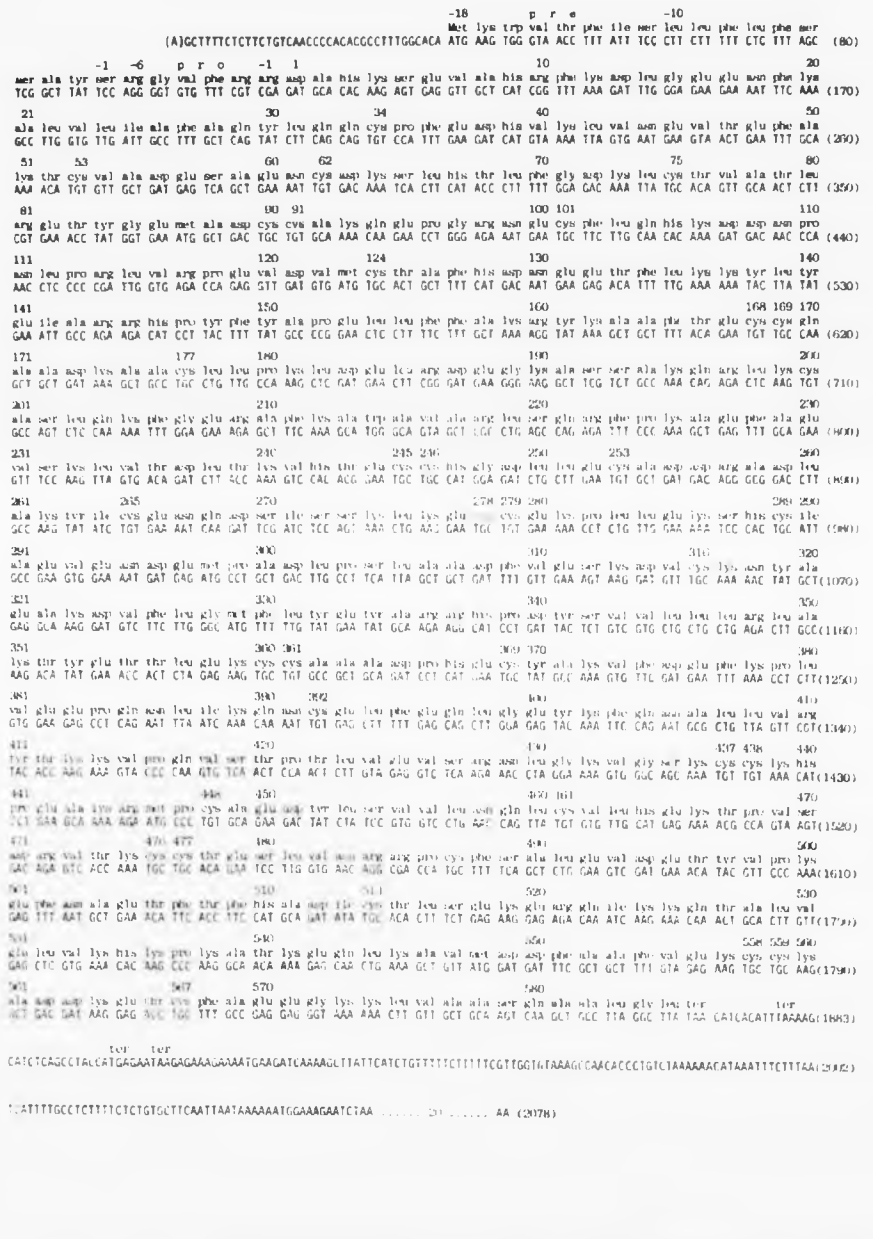


Fig. 1.3 The nucleotide and amino acid sequence of human serum albumin as determined by cloned cDNA[72].

1.3 THE STRUCTURE OF SERUM ALBUMIN

1.3.1 The primary structure of human serum albumin

The primary sequence has been determined by Behrens[24], Meloun[184] (Fig. 1.2) and lately by Dugaiczyk[72] (Fig. 1.3) who encoded the nucleotide sequence of HSA mRNA of cloned cDNA. Comparatively, the sequence of the latter two authors are in better agreement than that of Behrens[24].

However, some disagreement in the latter two sequences exist. These are listed below with the sequence determined by Meloun[184] in brackets:
95-gln (glu); 95-glu (gln); 97-gly (glu); 170-gln (glu); 464-his (glu); 465-glu (his); 501-glu (gln) and residues 364-370 are ala-asp-pro-his-glu-cys-tyr as (his-asp-pro-tyr-glu-cys-ala).

The latest sequence[72] has enabled one of the disagreements between the sequences of Meloun[184] and Behrens[24], concerning the absence or presence of amino acids between cysteines 278 and 279, to be settled.

The finding of an absence of amino acids has enabled the construction of a near-perfect homologous triplet domains as shown in Fig. 1.5

1.3.2 The secondary structure of serum albumin

The distribution of secondary structure of bovine serum albumin (BSA) is shown in Fig. 1.4. This

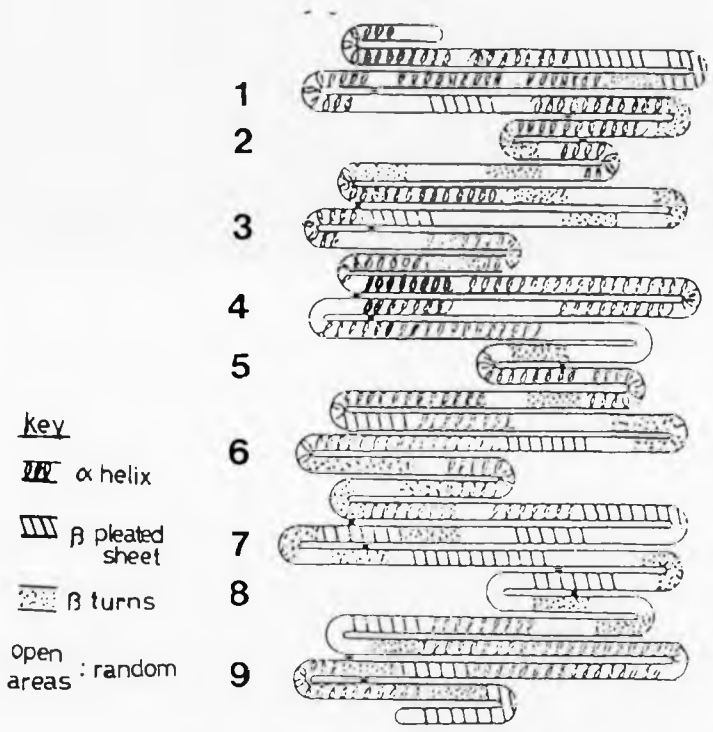


Fig. 1.4 The distribution of secondary structure in BSA, as calculated from the revised sequence of Brown[47] by rules of Chou and Fasman[60]. Residues 400-403 are assumed to be lys-phe-gln-asn[209].

structure, which was predicted by Peters and Reed[209], shows long α-helical loops modified with β-sheet and β-turns. These are most apparent in loops 1, 3, 6 and 7. At the tips of the loops are disturbances of the helices, and eight of the turns (seven of which involve cysteine residues) are predicted to be β-turns.

Brown's[47] model based on model building has six uniform helical regions, each of which consists of a long loop and a subsequent connecting piece. Other estimates, based on predictions from amino acid sequence and model building, shows a total of 46% α-helix and 16% β-sheet. This is in close agreement with the estimate from circular dichroism which is 50-55% α-helix and 15-18% β-sheet[205].

1.3.3 The tertiary structure of serum albumin

1.3.3.1 The Triplet Domain Model[47]

In this model albumin consists of a single peptide of over 580 amino acid residues arranged into a series of large (L) and small (S) loops (LSL-LSL-LSL-L) by cross-linking disulphide bridges between its 34 half-cysteine residues. However, a cysteine residue that should close a loop at 8 and 54 is lost, probably during evolution.

An adaptation of this structure by Dugaiczyk[72] is shown in Fig. 1.5. Each domain (LSL) consists of two subdomains each of which is formed by three α-helical rods 'X', 'Y' and 'Z'. The outer helices "X" and "Z"

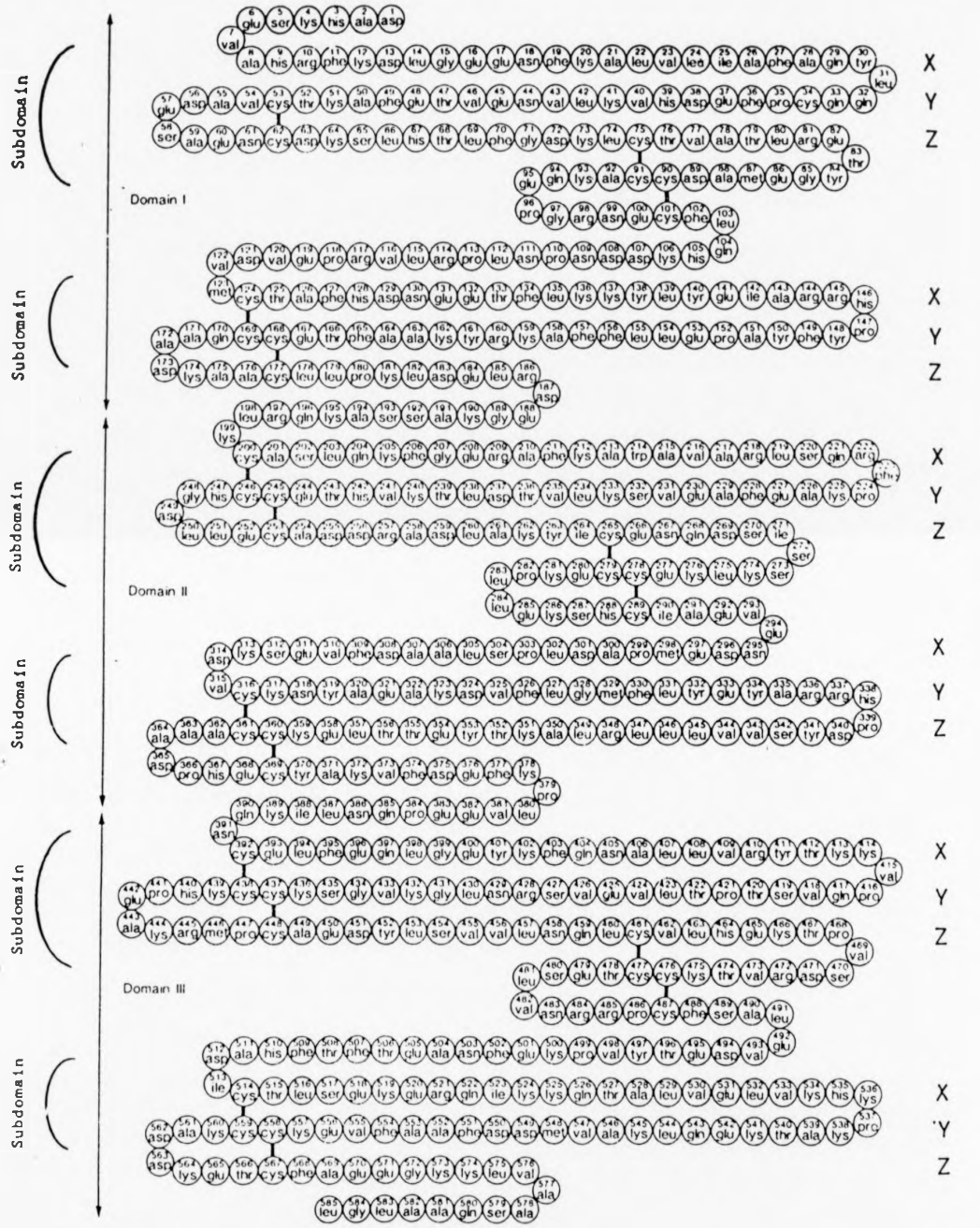
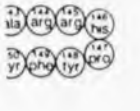
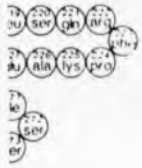


Fig. 1.5 Tertiary structure of human serum albumin[72]

Allocation of subdomain and helices, (X, Y and Z) are adapted from Brown[47].


X
Y
Z


X
Y
Z


X
Y
Z

X
Y
Z


X
Y
Z


X
Y
Z

umin[72]

(X, Y

are about 20 residues long and are attached to the middle 'Y' helix at one end by a disulphide bridge and at the other end by an invariant proline which causes a hairpin turn. These proline residues are strategically placed at the tip of each large loop (i.e. at 147, 225, 339, 416 and 537)[47].

The three rods are arranged into a trough-like structure to form a tailor-made ligand-binding site. The cavity of this binding site is lined with inprojecting hydrophobic amino acids with an abundance of basic amino acids localised at the entrance, e.g. arg-arg-his (336-338).

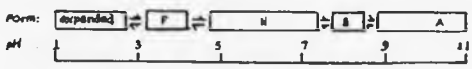
Structurally, the N-terminal position of albumin is relatively compact in comparison to the N-terminal portion. This may be due to the hydrophobic aromatic residues of loop 3.

The loop-and-link structure of albumin explains the ruggedness and flexibility of albumin. It can survive conditions fatal to other proteins such as extremes of pH, high salt concentrations (e.g. 6 M urea) and heat. This is because the local segments which are held in near-alignment by disulphide bridges can readily reform the secondary structure.

Other results to support the loop-and-link structure are the small and apparently independent fragments obtained by mild enzymatic cleavage. Each fragment consisted of an individual domain or a series of loops[206] which were well-ordered, contained about the same proportion of α-helix and still had the inherent ability to reform the original disulphide bridges, opened by

reduction in 8 M urea, into their three dimensional pattern as the parent molecule[220].

Another feature attributed to the loop-and-link structure is the reversible isomerisation of albumin at different pH values[316], perhaps due to the repulsion of loops as salt bridges are broken. The forms are as shown on Scheme 1.1.



Scheme 1.1 Isomeric forms of albumin at different pH values (F = fast migrating, N = normal).

The normal (N)-form predominates at pH 5-7 while the fast migrating (F)-form is formed below pH 4. Further expansion occurs below pH 3 to expose the hydrophobic residues.

In the alkaline range, the B form is produced at pH 8, especially in the presence of calcium ions. This form has a slight loss of helix and increased mobility of the thiol groups. Irreversible transformation occurs between pH 9 to 11 to the A form. Migration of this form is slower than the N form on electrophoresis, with an increase of 0.21 pI units[267]. In this transformation, the single thiol at cys-34 is implicated as a catalyst in effecting an interloop exchange of disulphide bonds. Such exchanges are said to be restricted to the first domain. The A-form has also been implicated as an intermediate in albumin degradation since it disappears faster *in vivo* than the N-form[293].

1.3.3.2 Earlier Conformational Models

These earlier models, which are now superseded by the Triplet Domain Model, were based on fragmentation studies and interpretation of isomeric transformations at acid pH values[117,84,88,204].

The latest of these models[204] as shown on Fig. 1.6 consists of four major segments of different sizes with a small arm projecting from the N-terminal end of the first segment. This was deduced from the cumulative enzymatic and chemical cleavage of unreduced (native) albumin[295,144,203].

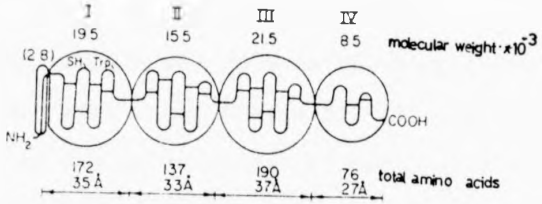


Fig. 1.6 Albumin model proposed by Peters[204].

1.4 ALBUMIN IN MAN

The normal level of plasma albumin is 35-45 g/l and is about 0.02 g/l higher in males than females[123]. The level is low in early development but rises to a high plateau at adulthood, and then decreases with increasing age. The average half-life is about 20 days[243]. Tryptophan incorporation appears to be the rate-limiting criterion in albumin synthesis[5] and this may function to conserve levels of this scarce amino acid.

Albumin is synthesised in the liver with an

additional peptide of 24 amino acids at the NH_2-terminal as shown on Fig. 1.3. The highly hydrophobic prepeptide of 18 amino acids is cleaved on secretion from the rough endoplasmic reticulum while the propeptide of 6 amino acids is removed from proalbumin by the golgi vescicle[208,283]. However, a point mutation in the propeptide does not appear to prevent the secretion of proalbumin into the circulation as is evident from reports of Proalbumin Christchurch[40] and Proalbumin Lille[1].

1.4.1 Chemical properties

The albumin molecule is the most anionic of the major plasma protein with a net charge of -19 at pH 7.4[271]. This high charge is due to an excess of carboxylic acids which is unequally balanced by lower levels of other amino acids, e.g. glycine, alanine, lysine, histidine and especially tryptophan. It has 102 carboxyl groups (pK 4.1), 15 imidazoles (pK 6.3), 1 α-amino group (pK 7.8), 56 ε-amino groups (pK 9.5), 17 phenolic groups (pK 10.2) and 23 guanidines (pK > 12). All the imidazole groups are accessible to hydrogen or hydroxyl ions in the pH range 2-12. About 50% of the carboxyls can be titrated in the normal (N) form with an intrinsic pK of about 4.3[86].

Spectral perturbation by bulky solvent molecules shows that 5-6 of the 17 tyrosine residues appear to lie at the surface at pH 7[121]. The single tryptophan in HSA and one of the surface tyrosines are located in an exposed location near the single sulphydryl group[193].

Numerous values for the isoelectric point of albumin, containing 2-3 moles of fatty acids are reported, such as pI_e 4.7[128] and pI_e 4.8[75]. The pI_e of albumin devoid of fatty acids and other warts are 5.6[75] and 5.2[128].

1.4.2 Heterogeneity of serum albumin

Isolated albumin is usually contaminated by a microheterogenous population of protein impurities, mixed disulphides, bound ligands, polymers, possible configurational and isomeric species, possible 'molecular ageing' and rare genetic variants[209]. Even the composition of purified HSA preparations can contain 0.03% N-acetylhexosamine[244], 0.02% trace metals[170] and 0.8% fatty acids[107] which, with vigorous defatting, can be decreased to 0.004%[58].

Common protein impurities in crude preparations are α-globulins and enzymes (such as an esterase which migrates slightly ahead of albumin). Mixed disulphides are formed from the reaction of albumins with the free sulphydryl group at cys-34 (mercaptalbumins) with cystine or cysteine to form non-mercaptalbumins[6]. Albumin sulphydryl group can also be found in higher oxidation states such as sulphonic acid[192].

Bound ligands are bilirubin, fatty acids, and can include nearly the whole range of pharmacopeia such as salicylates, sulphonamides, barbiturates, aureomycin, penicillins and Warfarin.

'Molecular ageing' can be due to modification of amino acid residues without breakage of peptide bonds, such as acetylation of ε-amino group by aspirin, or major

conformational changes[313]. Dimerisation increases with storage and fresh preparations contain about 5% dimers[82].

Albumin isomers are a well-known source of heterogeneity. Peterson and Foster[210] exploited their solubility differences to separate the F- and N-forms. The low-solubility of the F-and high solubility of the N-form at decreasing pH (5-3) is the main criterion of the solubility test of Foster[84]. A tertiary configurational change can be produced by 3-5 M urea involving either electrostatic or hydrogen bonds[113].

1.5 BINDING SITES ON ALBUMIN

1.5.1 Introduction

All the domains are dissimilar in hydrophobicity, net charge, ligand binding sites, and are functionally specialised.

The sites to which the multitude of substances known to bind to albumin can be classified as[209]:

(A) Hydrophobic, non-covalent sites for
  (i) primary long chain fatty acids,
  (ii) bilirubin and certain drugs,
  (iii) indole and certain other drugs.

(B) Covalent attachment for organic ligands including the thiol group.

(C) Chelation sites for divalent metals.

Fig. 1.7 shows the principle ligand binding sites on albumin.

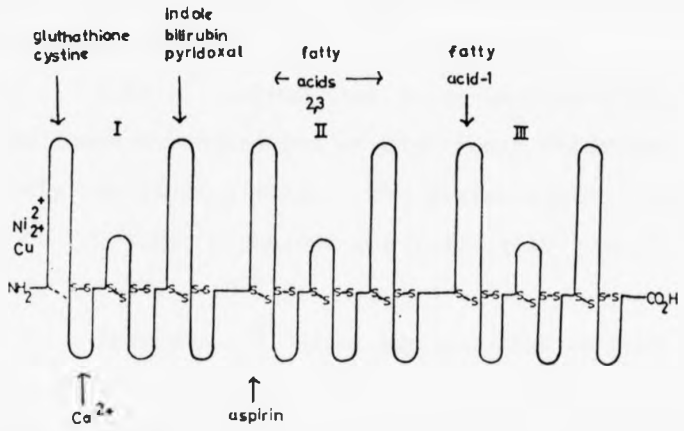


Fig. 1.7 Principle ligand binding sites on HSA.

Albumin is said to have allosteric properties where the assembly of 9 semi-autonomous loops can transmit information of conformational changes induced by binding of some ligands from one site of the molecule to discrete parts of the polypeptide chain[97].

1.5.2 The binding of bilirubin

1.5.2.1 Introduction

Albumin carries this breakdown product of haem to the liver for subsequent excretion. Failure in this secretory process can lead to uptake in the basal ganglia of the brain in jaundiced babies leading to kernicterus. The resulting neurotoxicity can lead to hearing loss, motor retardation and in extreme cases, death.

1.5.2.2 Binding Sites and Affinities

The studies of Beaven[20] and Jacobsen[133] on the

number of bilirubin binding sites and binding affinities are conflicting.

Beaven[20] showed that three binding sites exist at low ionic strengths but at high ionic strengths, there are only two binding sites. The dissociation constants (k_D) at low ionic strengths are 1.4-2 x 10^{-7} M, 3.3-10 x 10^{-6} M, and 3.3 x 10^{-5} M.

Jacobsen's[133] model has one site of high affinity (k_D = 7 x 10^{-9} M) and two of lower affinities (k_D = 2 x 10^{-6} M).

1.5.2.3 Location of Binding Sites

The location of the high affinity site is on loop 4 (residues 124-297)[151], and the second site is on residues 446-547[102]. However, Hutchinson and Mutopo[129] used photoactivated covalent binding of [^{3}H]-bilirubin to locate the binding site and their results showed that the majority of the label (62%) were in residues 1-24 and the remainder on residues 125-297. Hsia[127] found that the three bilirubin binding sites are specific, stereospecific and allosteric. Binding to the primary site altered the stereospecificity of at least one of the secondary sites.

Using chemical modification methods Roosdorp[226] showed that the primary bilirubin site contains a vital lysine residue but an earlier study implied that arginine, histidine and tyrosine were involved in binding at different sites on albumin[132].

When bilirubin and BSA react, two first order configurational changes take place after a primary complex is formed in a fast bimolecular step. A dynamic equilibrium is then set up between the primary, secondary

and possibly, the final product[148]. About 20-25 protons dissociate from bilirubin between pH 6 and 9 in this interaction[134], which has effectual similarities to that of temperature and calcium concentration on albumin[310].

Biliverdin IX-α only binding at the primary site of HSA (k_A = 1.3 ± 2 x 10^{-6} M^{-1}, defatted albumin; 1.3 ± 3 x 10^{-6} M^{-1}, pooled adult sera)[3].

1.5.2.4 Influence of Other Ligands

The binding of bilirubin is indifferent to the binding of nonesterified fatty acids even when 4-6 moles of fatty acids are bound per mole albumin to sites other than the bilirubin binding site[135]. Bile salts, hematin, sulphonamides, acetamides, salicylates, pyridoxal 5-phosphate, warfarin and numerous azo dyes compete for and decrease the effective binding capacity of bilirubin[204]. In an interspecies test, hematin was found to bind only to human and monkey albumin[51], probably by attachment to histidine residues[167]. The binding affinities to its specific sites on HSA were increased by addition of two moles laurate (C_{12}) per mole albumin, possibly by heterotropic cooperativity[43].

1.5.3 Binding of fatty acids

1.5.3.1 Introduction

Serum albumin serves as a sequestor of mainly unsaturated C_{16} and C_{18} free fatty acids (FFA) such as oleate, stearate, linoleate, archidonate (C_{20}) and palmitoleic in the circulation[255]. This increases the

concentration of fatty acids near the cell surface thus facilitating uptake by the cells. Under most conditions, the plasma FFA is only 2-4% of the total fatty acids present. These are then absorbed by adipose tissue for storage as triglycerides or released during shortage in the circulation[120]. The average half-life of a plasma FFA is only 1-2 mins. and they have a high turnover rate[92].

Albumin can bind noncovalently up to 2 moles of FFA without appreciably reducing its ability to bind a chemically different compound[257] and their presence is essential to the stability of the tertiary structure[128]. Addition of fatty acid anions facilitate the realignment of the secondary structure during reoxidation of fully reduced albumin[7]. Conformational changes which accompany the binding of these anions are detected by spectral changes at 295 nm[196].

1.5.3.2 Binding Models

Hydrophobic pockets in the clefts between the domains can provide ideal binding sites for fatty acids[256]. Each of the three domains could contain a structurally similar site in addition to two to four sites located inbetween two adjacent clefts[257]. The group of localised basic changes at the entrance to the cleft attracts the carboxylate group of the fatty acid while its hydrocarbon shaft is corsetted into the hydrophobic pocket[209].

Spector[260] formulated a Scatchard model containing three primary and three secondary sites while Goodman's model[107] had two primary and three secondary sites. In addition to these there are between 20 to 60 weak sites.

However, this Scatchard model does not accurately estimate the total number of binding sites and the binding constants obtained were non-specific. A new model based on Scatchard and equilibrium analysis showed that there are six strong binding sites and about 30 weak sites in tests using myristate (C_{14}) and palmitate (C_{16})[257].

1.5.3.3 Binding Affinities

The binding affinities of albumin decrease with decrease of the fatty acid chain length[107], that is, 14:0 < 16:0 < 18:1[15,16,258]. Insertion of one *cis*-double bond increases the strength of binding but insertion of a second double bond decreases binding below that of the corresponding saturated fatty acid[256], presumably due to the stereochemical factor.

1.5.3.4 Binding Sites

The primary binding site for palmitate is in the C-terminal domain loops 7-9 but chiefly near or in loop 7 (res. 377-582)[206]. Spector and Fletcher[257] suggested that one or more of the stronger binding sites are located near the hydrophobic cleft between the first and second cylindrical domain. This deduction was based on the observation of Spector and John[259] in that the tryptophan fluorescence of both BSA and HSA were progressively quenched when up to 5 moles of FFA are added. This could be due to a conformational change in the geographical region of tryptophan produced by binding of FFA at remote sites[256].

The primary binding site for medium chain fatty acid is located between fragments (124-297) and (298-585). The secondary binding site is in the same general region

as the primary site. Competition for binding of N-acetyl-L-tryptophan occurs at the primary site[164].

1.5.4 Binding of metals

1.5.4.1 Introduction

Albumin acts as a scavenger of heavy metal ions and thus prevents them from damaging the mitochondria[290].

1.5.4.2 Copper and Nickel

The nonceruloplasmin Cu(II) and Ni(II) ions of plasma are bound at a highly specific site on the N-terminal tripeptide. In HSA, this is NH_2-asp-ala-his. A tight chelate complex is formed by the first three nitrogen atoms of the peptide chain and one of the nitrogen atoms of his-3 to create a square planar ring into which a Cu(II) ion can fit snugly and the larger Ni(II) ion more clumsily[205].

A square planar or square pyramidal co-ordination geometry was detected about the Ni(II) ion. Its binding affinities are less than for the Cu(II) ion and the log (association constant) values for the binary and ternary complexes are 9.57 and 16.23 respectively[105].

His-3 is the key amino acid since the albumin of the pig and dog, which has a tyrosine in place of his-3, does not complex Cu(II) or Ni(II) ions in this manner. The identity of the first two amino acids are unimportant for chelation as shown by bovine and rat albumin.

The secondary Cu(II) ion binds at the single sylphydryl group to form a dimer analogous to the mercury dimer, albumin-S-Cu-S-albumin[49].

1.5.4.3 Other Metals

Monovalent Ag(I) ions bind with high affinity but cannot cause dimer formation. The binding of metal ions other than Cu, Ni and Hg are of lower affinity, more electrostatic in nature, and react similarly as other proteins with metals[111]. Binding of metal ions increases with pH and is minimal below the isoelectric point of albumin.

1.5.5 The binding of drugs and other ligands

1.5.5.1 Introduction

Drugs are an important and diverse group of substances that bind to albumin. Although diverse in chemical character, the tightly bound drug usually contains a strongly electronegative centre or is an organic acid[247]. These are bound to HSA on strongly basic sites by electrostatic attractions.

The binding affinity of a drug is significantly influenced by the pharmacokinetics of the drug. When it is tightly bound ($k_A > 10^5\ M^{-1}$) to HSA, more than 95% of the drug will be concentrated in the blood and interstitial fluid leading to an immediate decrease in pharmacological value of the drug[139]. However, in most cases, competitive binding by other substances will displace the bound drug[250]. The therapeutic concentration of a drug is such that the molar ratio of a drug:albumin seldom exceeds 1. There is little or no competition between FFA and other drugs for binding sites so long as the FFA concentration remains below 1 mM[107]. The blood plasma

concentration of FFA is relatively low, between 0.3 and 1.2 mM[257].

Drugs are known to bind at specific pre-existing sites of different specificity on the albumin molecule. However, the versatility of albumin may also include conformational induction to form specific sites. There is evidence to support the mutual cooperative effects between binding sites and the independent binding of drugs, but it is difficult to distinguish between simple direct competition and cooperative effects[97].

1.5.5.2 Location of Binding Sites

The specific location of the binding site of a particular drug can be elucidated by affinity labelling, followed by enzymatic or chemical cleavage; and/or by the binding interaction (e.g. inhibition, or competitive displacement) between a variety of compounds. The majority of drug binding sites are located in loops 3 and 4 of the albumin molecule[24].

Binding at specific sites or interaction with a certain moiety on the albumin is exemplified below. In most cases, basic residues inherent in the albumin sequence interacts with the electronegative moiety in drugs. Substitution of the strongly electronegative centre of the drug or chemical modification of the basic residues on albumin results in loss of binding properties[44].

Warfarin (1) binds at a single, high affinity, site (k_A = 2.5 x 10^5 M^{-1}, pH 7.4) at pH 6.9 and is higher in the basic conformation of albumin than the neutral. The high affinity constant is increased by calcium ions

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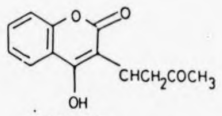
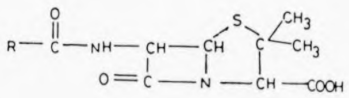
but chloride ions probably displace the bound drug as shown by reduction of the induced elliptic conformation and fluorescent intensity[139,309]. The binding of warfarin is stereospecific and enantiomers can be distinguished[48].



WARFARIN

(1)

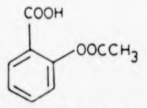
Penicillin G (2) binds through an amide-type linkage between the ε-amino groups of lysine residues and the carbonyl carbon of the functionally active β-lactam ring[52].



PENICILLIN G

(2)

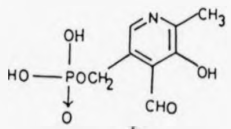
Other drugs that interact with basic residues on albumin are acetyl salicyclic acid (aspirin) (3) which acetylates the ε-amino group of lys-199 located at the base of loop 4[118];



ACETYL SALICYLIC ACID

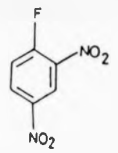
(3)

pyridoxal 5'-phosphate (4) which forms a Schiff base with lys-223 of BSA[8]; 2,4-dinitrofluorobenzene (5) and 2,4,6-trinitrobenzene sulphonate (6) link covalently to lys-222 of BSA[282];



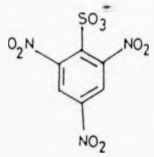
PYRIDOXAL 5'-PHOSPHATE

(4)



2,4-DINITROFLUOROBENZENE

(5)



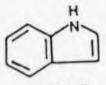
2,4,6-TRINITROBENZENE SULPHONATE

(6)

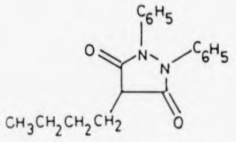
cyclohexanedione is said to bind arg-145, arg-218 and arg-222 in HSA[248] and the highly electronegative atom at C-7 of benzodiazepine binds at a vital arginine residue[247].

L-tryptophan binds exclusively to albumin. There are one high affinity and two secondary sites on HSA and BSA[249] and binding is not influenced by non-esterified fatty

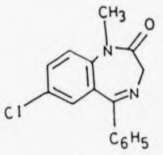
acids[264]. At the high affinity site, maximal binding (k_A of about 2 x 10^4) occurs at pH 9[180] and Fehske[76] showed that a highly reactive tyrosine residue is part of the binding site. Gambir[96] found the indole (7) binding site to be located at his-146 and lys-190 in loop 3. Phenylbutazone (8) and diazepam (9) compete or bind at this site.



INDOLE



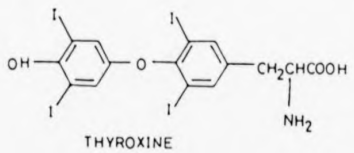
PHENYLBUTAZONE



DIAZEPAM

(7) (8) (9)

Thyroxine (10) is bound to albumin only when binding to the thyroxine-binding globulin is over-subscribed[25] at one strong binding site[265] with k_A of 10^6 [113]. High resolution x-ray studies showed that tyroxine is bound to one or two identical sites in a narrow cylindrical channel running through the long axis of prealbumin[31]. Tyrosine, tryptophan fatty acids, dinitro phenol and methyl orange (12) compete for binding at this site[286].

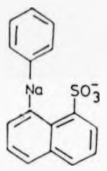


THYROXINE

(10)

Binding of albumin to prostaglandin H_2, the endoperoxidase intermediate of prostaglandin syntheses[168] has been implicated in the inhibition of prostaglandin biosyntheses *in vitro*[232].

Dodecyl sulphate binds at 8-9 sites with an average k_A of 1.2×10^6 (M^{-1})[221] and the fluorescent probe ANS (8-anilinonaphthalene-1-sulphonate)(11) binds at 5 sites in the hydrophobic regions of albumin[294]. The sites



1-ANILINONAPTHALENE-8-SULPHONATE

(11)

appear to be on residues 1-386. N-acetyl-L-tryptophan competitively displaces one of the bound ANS molecules[98].

1.5.6 The binding of dyes

1.5.6.1 Introduction

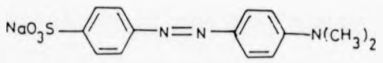
The binding of dyes to albumin is used in physiochemical, medical and analytical techniques. Dye binding induces configurational changes and they can protect the albumin molecule against enzymatic hydrolysis and denaturation[173].

The tetrabromo-derivatives, bromocresol green[225] (16), bromophenol blue[30] (13), bromosulphonaphthalein[19]

and phenol sulphonaphthalein (Phenol Red)[224], bind with an average affinity constant of about 10^{6} M^{-1}. The other naphthalene sulphonates, e.g. Congo Red (18), Tryphan Blue, Evan Blue and azobenzoates (e.g. Methyl Red) are tightly bound[52]. Schneider[241] showed that the rate of access to the organic ligand site is entropy controlled.

1.5.6.2 Dyes used in Estimation of Albumin

The three most commonly used dyes in albumin assay are HABA [(2-4'-hydroxyazobenzene)benzoic acid][191] (15), methyl orange[142] and bromocresol green[70] (16). Since methyl orange gives dubious results in nephritis[142], and the binding of HABA is temperature-sensitive and affected by bilirubin and certain other drugs (e.g. heparin, salicylates), bromocresol green is now used in clinical estimations of albumin.



METHYL ORANGE

(12)

Bromocresol green also binds to α- and β-globulins[296] and in BSA the binding of numerous dye molecules is shown to be loose with only a few specific binding sites. The binding of the numerous dye molecules is proportional to the size of fragment as shown on Fig. 1.8.

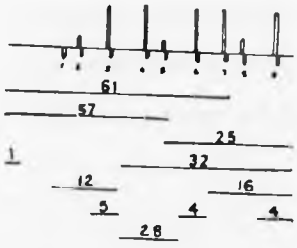


Fig. 1.8

Binding of bromocresol green to isolated fragments of BSA (figures are percent of colour change observed with an equimolar amount of BSA)[206].

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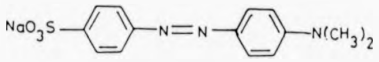

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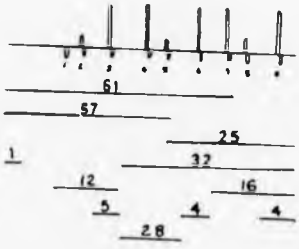

Fig. 1.8

Binding of bromocresol green to isolated fragments of BSA (figures are percent of colour change observed with an equimolar amount of BSA)[206].

For use in analytical estimations, Gustafsson[112] found that bromocresol green binds almost instantaneously to albumin, in between 6-30 seconds. Globulins are only bound from 10 to 15 mins. Also, since this albumin-dye complex absorbs at 615 nm, interference by bilirubin and haemoglobin can be avoided.

Rice[222] used ANS (11) to determine albumin concentration since the fluorescence obtained is highly specific and sensitive.

1.5.7 The binding of antigen

1.5.7.1 Introduction

Antigenic sites on albumin are binding loci whereby the antibody is regarded as the macromolecule and albumin as the ligand.

1.5.7.2 Characterisation of the Antigenic Site

Characterisation of the antigenic binding site usually involves the isolation of intact antigenic site on fragments cleaved by enzymatic or chemical scissors. The ensuing detection may be by combining the fragment to the antibody and therefore preventing it reacting with its normal antigen. However, because of the heterogenous population of antibodies produced by immunisation and the multitude of different binding sites on the antigen, the fragment will bind the antibodies specific for the site it represents, but the rest of the antibody directed against other sites, will still be able to form a precipitate.

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1.5.7.3 Binding Sites

Horse albumin has 8 antigenic sites and the molar ratio of albumin to antibody is 4. There are 2 binding sites on each IgG molecule[119]. Lapresle[158] showed that in HSA there are at least three different antigenic determinants. This result was obtained in fragments cleaved by cathepsin at pH 3.5, pepsin pH 1.4, and chymotrypsin at pH 4.5. Loop 9 of HSA has two strong binding[27] sites in the C-terminal region[208].

Double immunodiffusion of bovine albumin and its various fragments against rabbit anti-albumin showed a progressive loss of determinants in fragments of decreasing size obtained from either the N- or C-terminal ends. The two halves of bovine albumin can recombine to regenerate a complete antigen[27].

1.5.7.4 Specificity of Binding

The specificity of the antigenic site on albumin is a subject for debate. Lapresle[156,158] found the antigenic sites to be specific and distinct. On the contrary, Atassi[17] and Habeeb[115] found homologous, repeating antigenic sites. A recent report by Doyen[71] appears as a compromise. Cross reactivity between antigenic sites are found such that an antibody specific for an antigenic site can cross-react with a site on another part of the molecule. Since albumin contains 18% homology[46], a certain degree of duplication can be expected. The specificity of the isolated antibodies were shown to react with the fragment used for their isolation (homologous) but also with other fragments (heterologous). However,a relatively high concentration of antibody was needed to cross-react

with heterologous antigenic sites. This is in agreement with predictions of Peters and Reed[209].

Carboxylate groups are essential at the antigenic site of albumin. Mild esterification inhibits the precipitation of albumin with specific antisera but esterified albumin can still form soluble antigen-antibody complex so that at least one site may not include a carboxylate group[262].

1.6 ALLOALBUMINEMIA

1.6.1 Introduction

Alloalbuminemia can be defined as the rare occurrence of either two forms of albumin (bisalbuminemia), or one variant form of albumin, in the serum. In bisalbuminemia, the mobility of the variant albumin on electrophoresis is either slightly advanced or retarded with respect to the normal allotype. Single variant albumins are usually difficult to detect unless a comparative parallel run with the normal allotype is made on electrophoresis. This difference in mobility of allotypes is attributed to the difference in total charges of the albumin molecule.

Bisalbuminemia (synonyms: paraalbuminemia, alloalbuminemia, dialbuminemia, isoalbuminemia, double- and split-albuminemia) can be classified into two categories.

I Congenital hereditary bisalbuminemia

(i) Structural bisalbuminemia arising from

point mutation(s) in the primary amino acid sequence.

(ii) Albumin dimers arising from noncovalent and/or disulphide interactions between two structurally similar albumins.

II Non-hereditary transient bisalbuminemia

Arising from attachment of small organic ligands to normal albumin or as a consequence of pancreatic disease.

1.6.2 Congenital hereditary bisalbuminemia

The occurrence of double albumin bands is a congenital condition, transmitted by heterozygotic individuals, whereas in the case of a homozygotic carrier only the variant allotype is represented. This genetic transmission is by a non-sex linked, autosomal, codominant mechanism where the phenotypic expressions of both alleles are equal. The quantitative ratio of the normal:variant albumin and the sex ratio of the affected individuals are nearly always near equality and these characteristics support the codominancy theory of the albumin gene.

The gene symbol for the normal albumin, albumin A or (A_1) is Al^A (accepted notation) = Al^N, the slow allotype (A_2, B) is Al^S and the fast allotypes is Al^F. Therefore, heterozygotes for the slow and fast variants are Al^A/Al^S and Al^A/Al^F and homozygotes are Al^S/Al^S and Al^F/Al^F respectively. Heterozygotes for two different variants have never been reported.

Variants are named for ethnic, geographical, or laboratory origin, their gene symbols being abbreviations of their names. Exceptions to this rule are albumin B, reserved for the most common allotype found in Europeans initially detected by Earle[73], and several Italian cases named for the location of the laboratory and place of origin of the proband, using Italian car number plates for their gene symbol.

1.6.2.1 Mechanisms of Mutation

The variant albumin arises from mis-sense point mutations where one or several amino acids are substituted at given sites in the primary amino acid sequence of albumin. The substitution may be a transition (pyrimidine-pyrimidine or purine-purine substitution) formed as a result of inversion of a particular cistron segment, or a transversion (purine-pyrimidine substitution) in the DNA[263].

An example of a transition (guanine to adenine substitution) is the replacement of glutamic acid -570 (coded by GAA or GAG) by lysine (coded by AAA, AAG) in albumin B[100]. Another example of a transition (guanine to adenine substitution) is Proalbumin Lille[1] where arginine (CGU, CGC, CGA, CGC) is replaced by histidine (CAU, CAC). The unnamed variant of Gentou and Plazonnet[99] exemplifies both transition and transversion substitutions. In this case, lysine (AAA, AAG) replaces glutamic acid (GAA, GAG) in a transition and alanine (GCU, GCC, GCA, GCG) replaces proline (CCU, CCC, CCA and CCG) in a transversion. Most of the determined amino acid mutations

in albumin variants, except for Albumin Naskapi (Section 1.6.2.9.3), are unaffected by the revised HSA primary sequence of Dugaiczyk[72] (Fig. 1.3).

1.6.2.2 The Albumin Gene

The albumin locus is highly variable in man[237], where it can produce a line of allotypes[197] and is extremely close to the Gc locus [Hirschfield's[124] group specific α-glycoproteins with phenotypes Gc 1-1, Gc 2-2 and Gc 1-2 on the same autosomal chromosome [140], with a map distance of 1.5% between them. The linkage probability was calculated by Smith[251] to be 0.9788. The close proximity of these two loci are borne out by the recombination values for a number of affected families bearing different albumin variants.

Lau *et al.*[160] showed group specific segregation of Albumins B and Gainsville with Gc-2 and that albumin Naskapi frequently segregates with Gc-1 but rarely with Gc 2-2 Some strongly cathodic variants occur with all three common Gc phenotypes[297].

1.6.2.3 Alloalbuminemia in Man and other Animals

1.6.2.3.1 Animals

The autosomal and codominant character of the albumin alleles can be demonstrated by controlled genetic studies in animals, especially hybrids. These studies show that three phenotypes of Al^F and Al^S are possible, that is the heterozygote is Al^F/Al^S and homozygotes are Al^F/Al^F and Al^S/Al^S 37,179,268.

In horses[268] and sheep[287], the three dominant alleles are A, B and C. Tne appropriate cross-

progeny produce the predicted homozygous (AA, BB, and CC) and heterozygous (AB, AC, BC) phenotypes.

The O alleles control highly diminished albumin production so that pigs with phenotype OO are analbuminemic and phenotypes AO and BO have only half the normal albumin levels[150]. Albumin polymorphism also occurs in some breeds of cattle[253], horses[62], and toads[114].

1.6.2.3.2 Man

In man, the first observation of double albumin bands was by Scheurlen[240] in the serum of a Swiss-German diabetic patient. However, it was later that the hereditary aspect of heterozygotic alloalbuminemia was observed by Nennstiel and Becht[189] and Knedel[147], who coined the term 'double-albuminemia' and designated the two albumins 'A_1' and 'A_2'. Simultaneously, Earle[73] found a similar albumin anomaly, which he termed 'A' and 'B', in 25 individuals of a pedigree of 58 persons. Dimeric variants were first reported by Fraser[91]. These initial reports of a rare and unusual anomaly generated much medical interest because of the extreme rarity of these genotypes in Caucasians. However, this general opinion was to change by the exposure of a polymorphic variant, Albumin Naskapi, by Melartin and Blumberg[182] in 1966, amongst the Algonkian-speaking people of North America. This variant is yet to be found in Caucasians, Negroes or South American tribes. From this and other studies, alloalbuminemia appears to be more interesting anthropologically rather than medically.

Another medical expectation was the extreme

lethality of the homozygous gene (e.g. Al^{Na}/Al^{Na}) and a double dose of this rare gene was expected to result in dire clinical consequences. Adams[2] expected it to occur as a result of consanguineous union. On the contrary, all if not most homozygotes are healthy.

1.6.2.4 Hypotheses on Alloalbuminemia

Since alloalbuminemia is not associated with any apparent diseases or symptoms, one wonders why nature has safeguarded the survival of this gene. Could it also be possible that the aberrant albumin is evidence of an evolutionary progression of a protein that is redefining its properties in an increasingly chemical world? For instance, several named variants have binding properties distinguishable from the normal allotype and mutation rates are high in culturally isolated societies that are adjusting to the modern world.

In 1967 Franglen[88] suggested that two albumins are present in normal individuals and both are electrophoretically similar. However, in bisalbuminemia, an aberrant albumin leads to the formation of an electrophoretically different variant. On a similar theme, Gitlin and Gitlin[101] suggested that 'normal' albumin is actually polymorphic. In this case the aberrant 'normal' albumin has substitutions between neutral amino acids. Bergstand and Czar[28] suggested that slow albumin was due to continue production of a normal foetal protein (α-feto protein?), and these are limited by the maternal immune system[108], but this suggestion was shown to be unlikely due to an increasing understanding of the subject.

Evidence to support these postulates is the abnormal binding of thyroxine by Al A in a bisalbuminanemic patient[236]. However, it can be argued that thyroxine is not an obligate ligand and is bound to albumin in times of saturation in the plasma. Yamamoto[315] obtained almost equal binding (17.0 + 31.5 μg T_4/g albumin) of both normal and slow variants.

Mutations in albumin have been found to occur especially frequently in Amerindian population and indeed Neel[187] estimated the mutation rates of South American Indian tribes to be eight times that assumed for other populations as attested by their high rates of chromosome breakage, mercury intolerance and high susceptibility to diseases.

The design of a new variant albumin may be to decrease its binding capacity for their culturally accepted but harmful drugs, e.g. hallucinogens and narcotics (these drugs may be mutagens) and to increase its adaptability to withstand modern day diseases, chemicals and so ensure their survival and reproducibility.

1.6.2.5 Methods of Classification

The majority of albumin variants are detected in hospitals and by the multi-disciplinary protein-typing of certain racial groups or tribes by electrophoretic procedures. However, electrophoretic procedures cannot detect all protein variants at the amino acid level. The number of alleles undetectable by electrophoresis is calculated to be proportional to population size[246]. The pH of electrophoresis is important. Although most

routine hospital electrophoresis is at about pH 8.6, fast variants are more clearly resolved at pH 5.4[242] and identical allormorphs to albumin A either cannot or are extremely difficult to detect by simple conventional electrophoresis, but not by peptide mapping.

1.6.2.5.1 Standard Method of Classification

There are two standard methods of classification.

I Vertical starch gels system of Weitkamp[297]

The buffer systems are acetate-EDTA (pH 5.0), *tris*-lithium-succinate-citrate (pH 6.0) and *tris*-EDTA-borate (pH 6.9). These pH values were chosen for maximum discrimination between slow and fast variants. The relative mobility of the variants are different over this pH range as shown on Table 1.1.

TABLE 1.1. *Relative mobility of 25 "monomeric" albumin variants in three starch gel electrophoretic systems*[a]

Variant	*Buffer system*		
	pH 5.0	*pH 6.0*	*pH 6.9*
1. RSI	1	2	6
2. Pollibauer	1	2	4
3. Belem I	2	1	4
4. B	3	3	1
5. Roma	3	1	3
6. Gainesville	3	3	4
7. Paris (Gombak)	4	3	5
8. Kashmir (Afghanistan)	5	2	1
9. Otsu	6	4	2
10. Santa Ana	7	4	3
11. SO BS	7	5	3
12. Cartago	7	3	5
13. Naxante	8	6	7
14. Pushtoon	8	4	6
15. Cayemite	9	5	6
16. Mexico	9	4	5
17. Uimba	9	6	7
18. Yanomama-2	10	6	6
Normal albumin	10	6	7
19. Medan	11	6	8
20. Maku	12	7	10
21. New Guinea (Reading)	13	9	9
22. Makiritare-3	13	8	9
23. Naskapi	14	10	10
24. Gent	15	11	10
25. Kyoto	16	11	10

The variants with mobilities closest to normal albumin (no. 13-21) are found in non-European populations especially Indians. Wider separation is seen in European variants.

Actual reversals in mobility occur, for example, albumin Máku migrates faster than Naskapi in the pH 5.0 system but not as far in the pH 6.9 system[301].

II Test schedule by Tárnoky introduced at the Royal Berkshire Hospital[274]

This involves tabulating the relative electrophoretic mobility of the variants on a 0-100 scale (0 = cathodic trailing edge and 100 = leading edge of albumin A) by the electrophoretic following systems.

(i) Whatman 3 MM paper in barbitone buffer (pH 8.6, 0.05 M).

(ii) Cellulose acetate (Shandon Celagram cellulose acetate strips) in barbitone oxoid buffer (pH 8.6, 0.1 M).

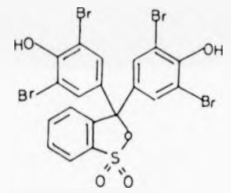
(iii) Helena Titan III zip zone cellulose acetate plates in barbitone buffer (pH 8.8, 0.05 M).

(iv) Disc-polyacrylamide (7%)[277] in a discontinuous buffer system (pH 8.3 → 8.9 → 8.3).

(v) Agar (1%) in barbitone buffer (pH 8.6, 0.05 M)[130].

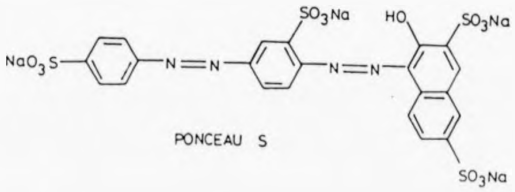
(vi) Agarose (1%) containing sucrose (5%) in barbitone buffer (pH 8.6, 0.065 M) [Corning ACI electrophoresis system].

The comparative ability of the variants to bind ligands were tested by incubating serum with the six dye solutions (approximately 250 µg/ml serum): bromophenol blue (13), Ponceau S (14), 2,4-(hydroxyazobenzene)benzoic acid (HABA) (15), bromocresol green (16), bilirubin (17), and Congo Red (18) at 37°C for 10 min. before electrophoresis on all six media. All except HABA were added to an excess of albumin.



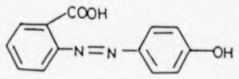
BROMOPHENOL BLUE

(13)



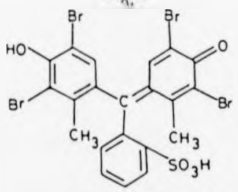
PONCEAU S

(14)



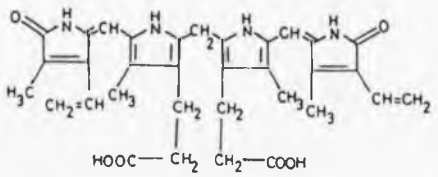
(2-4'-HYDROXYBENZENE)BENZOIC ACID

(15)

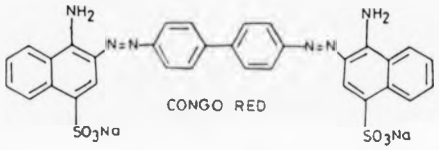


BROMOCRESOL GREEN

(16)



(17)



(18)

1.6.2.5.2 Critique of Ligand Binding Methods

The binding of fatty acids and dyes to albumin A are described in Sections 1.5.3 and 1.5.6. In these tests the pre-existing and inherently-bound ligands are not leached out to ensure true quantification of these test ligands, for example, sera obtained from patients may have drugs already bound to them and these may bind at or block the binding of these test ligands. Sera also nearly always contain a proportion of bilirubin bound to albumin. This binding is tight with k_D in the region of 7×10^{-9} M [133] (Section 1.5.2.2).

Although differential binding of ligands to albumin variants is well-used in proving non-identity of newly detected variants, this analysis is affected by the reversible binding of ligands during electrophoresis.

During electrophoresis, ligands may remain bound to albumin because their binding affinities are greater than the field strength at the electrodes. After an interim period, ligands become detached because of their faster electrophoretic mobility and can migrate as a separate band. If quantification of a strongly anionic ligand were taken, the fast band of a fast variant and the normal band of a slow variant would appear to bind more ligands. This can be offset by finding the optimum length of electrophoresis in a stated field strength.

Reversible binding of bromophenol blue occurs during isoelectric focusing of this variant Albumin Munday. The dye, added as a marker to follow the migration process, becomes detached when the albumins are focused and travel as one band to the anode.

Although not implying the same situation, albumin A in numerous slow variants, such as Albumins Vellore and Kashmir, bind more bromophenol blue than the slow allotypes[122]; but the fast allotypes, e.g. Albumin Reading bind more bromophenol blue[279]. Tárnoky[278] proposed that, in general, fast variants bound more bromophenol blue than the slower variants but Wieme[307] showed that this observation can be dispelled by the inclusion of bromophenol blue into the electrophoretic media to compensate for its reversible binding during electrophoresis.

On the other hand, undefatted albumin ensures a stable tertiary structure. This outweighs the advantages of an unstable albumin molecule, depleted of stabilising fatty acid anions (Section 1.5.3.1), with possible deformed

binding sites. The IFCC criterion for standardisation of albumin preparation is 1-2 moles of fatty acid per mole of albumin with a polymer content of less than 1%[275].

Non-esterified fatty acids do not interfere with bilirubin binding (as used in this classification) even when a physiologically large number of fatty acids (4-6 moles) are bound to a mole of albumin[132].

Even if dyes were to compete for fatty acid binding only one or two of the high affinity sites are 'permanently' occupied leaving the remaining high affinity and weak sites available[257]. The detection of differential binding of dyes used in standard quantification of albumin by variant albumins has led to their reassessment of their values.

1.6.2.6 Other Methods of Classification

1.6.2.6.1 Binding of Ligands

I Panel of Ligands Cavalli-Sforza[55] devised a method of screening serum or plasma for variants based on a panel of 63 radioactive ligands which include vitamins, hormones, drugs, amino acids, purine, pyrimidines, sugars and lipids. Binding was then deduced from PAGE-autoradiography.

II Binding of Thyroxine Binding of [^{131}I]-thyroxine by variant albumins is mainly quantified by autoradiography or scintillation counting.

1.6.2.6.2 Stability of Alloalbumin to Freezing and Thawing[130] The sera is frozen to -20^{o}C and rapidly thawed at 37^{o}C ten times. Its electrophoretic mobility is then compared to the control sera.

II High Temperature[11] This involves heating sera to 56°C for 30 min. and 2 hr. Schultz and Hereman[243] found an alloalbumin unstable to storage at room temperature. The slow Spanish variant, Albumin Luarca, appears to be dimeric rather than separate unique proteins since it merges into albumin A on freezing, thawing and storage at room temperature and the proportion of variant albumin is decreased by 9% (i.e. from 44% to 35%)[131]. This might imply that this 'slow' variant is formed by the increased binding of cations which are then detached by repeated freezing and thawing.

1.6.2.6.3 Molecular Weight

The monomeric or dimeric nature of variant can be easily elucidated by gradient-PAGE. This method supersedes ultracentrifugation methods.

1.6.2.6.4 Immunochemical Character

The immunochemical character of the variant albumin can be detected by its reaction against specific anti-albumin A and/or reaction of albumin A against specific anti-albumin variant. However, this has never been analysed employing the very sensitive, unique monoclonal antibodies.

Most varients are concluded to be immunochemically identical to albumin A because of several contributing features: the repetitive homology of the albumin molecule leading to numerous near identical binding sites (Section 1.5.7.4); secondly, the site and character of the mutant amino acid may not be involved or contribute

drastically to antigenic charcter and thirdly, binding at only a few sites may be sufficient to form the antibody-antigen precipitate. Even if the mutations were involved in antigenic specificity, the methods may be insufficiently sensitive to detect any microscopic differences in the variants, so that they appear to be immunochemically similar.

Albumin Chiari (BS/VR) was classed as immunochemically similar to albumin A by the reaction of normal and variant albumin against anti-normal albumin and anti-bisalbuminemia antibodies[4].

Robbin[223] and Margni[172] have, however, found inconclusive differences in antigenic specificity. Robbin[223] reported two immunoprecipitation areas formed by cross-reaction with diluted (1:30) variant sera. They attributed this to a second precipitation with excess antibodies without ruling out slight immunochemical differences with albumin A.

Margni[172] immunised rabbits against separated fractions (I and II) of the albumins. They found that anti-albumin-I antibodies bound albumin-II, but anti-albumin-II reacted with albumin-I bound albumin-II only slightly. This phenomenon may be explained by a depletion of antigen binding sites for albumin-II.

1.6.2.6.5 Isoelectric Point

Analytical isoelectric focusing could be useful in the classification of variants but isoelectric points are very rarely determined. Some of the objections include the possible binding of ampholytes carriers by albumin[85]

and even normal albumin has several published isoelectric points (Section 1.4.1). However, like any empirical study, the standardisation of this technique should enable precise comparisons to be made.

Bradwell and Hornbeck[39] used pH-gradient elution on the strongly anionic ion-exchanger, QAE Sephadex, to determine the isoelectric part of slow and fast albumins. However, reproducible pH elution profiles are very difficult to generate and some variants are sufficiently similar to albumin A to hinder separation, let alone to determine its isoelectric point.

1.6.2.6.6 Proportion of Normal to Variant Albumin

Most monomeric variants occur in a 1:1 ratio to normal albumin. Some reported exceptions are:

Name of Variant	Normal:Variant	Reference
Genoa /Catania	9:1	214
Cuneo/Belluno	8:2	289
RIH	6.4:3.6	56
Stirling	6.6 → 5.9:3.3 → 4.1	67
Vancouver	3.5:6.5	93
B	6.2:3.8	66

Most dimers constitute less than half of the toal albumin, e.g. albumin Warao is 30% of total albumin[11].

1.6.2.6.7 Mutation Point(s)

Tárnoky[274] proposed that the mutation points will be the ultimate method of classification. The

and even normal albumin has several published isoelectric points (Section 1.4.1). However, like any empirical study, the standardisation of this technique should enable precise comparisons to be made.

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1.6.2.6.7 Mutation Point(s)

Tárnoky[274] proposed that the mutation points will be the ultimate method of classification. The

location of mutations of only a few variants have so far been determined.

1.6.2.7 The Population Distribution of Variants

Albumin variants discovered by mass investigations or by hospital screenings have been categorised according to their gene frequencies and geographical distributions[238] or according to population frequencies[279].

However, this categorisation cannot be a true representation of the total occurrence of these rare variants because of the dependence on availability of resources such as equipment and finances. One need only compare the situations found in the well-developed Western World where facilities are readily available to that of selected mass-screening of small indigenous tribes and the limited screening in the poor Third World countries where financial priorities are given to more urgent needs for survival.

So far about 80 variants are named of which about 28 types can be distinguished. The distribution of some known variants (Table 1.2) shows a fairly orderly distribution. These can be divided into three groups. The first group are private variants restricted to one particular family, e.g. Albumin Cayemite[299]. The second group encompasses rare variants found in two or more vastly different ethnic cultures, e.g. Albumins Reading[279] and New Guinea[304], or are distributed widely regionally, e.g. albumin B[73,297]. The last group are polymorphic variants restricted only to the ethnic group or to some neighbouring groups, e.g. Albumins Naskapi[182] and Mexico[90].

Table 1.2 The population distribution of some albumin alleles - Adapted from Weitkamp[303]

Race	Variant	Population
Caucasoid	Pollibauer	Austrian descent (Holland)
	B (SO/CZ)	Swiss, Norwegian, Danish, Swedish, German, English, Italian
	Roma	Italian
	Gainesville	English, Irish descent (USA)
	Paris (Gombak)	French
	SO/BS	Italian
	Cartoga	Spanish (Costa Rica)
	Reading (New Guinea)	British, Greek, Swiss descent (USA)
	Gent (MI/fast)	Danish, Belgian, Italian
	"Dimer"	Welsh, Swedish
Negroid	Cayemite	Negro (Haiti)
	Uinba	New Guinea, indigenes
	New Guinea (Reading)	New Guinea, indigenes
	"Dimer"	Negro (USA)
Mongoloid	Gombak (Paris)	Indonesian
	Kashmir (Afghanistan)	Pushtoon (Afghanistan)
	Pushtoon	Pushtoon (Afghanistan)
	Mexico	Indians of South Western USA and Mexico
	Medán	Malayan aborogine
	Máku	Yanomamö (Venezuela), Makiritare (Brazil)
	Makiritare-3	Makiritare (Brazil)
	Naskapi	North American Indian
	Makiritare	Warao, Makiritare (Venezuela), Trio, Wajana (Surinam)

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	Reading (New Guinea)	British, Greek, Swiss descent (USA)
	Gent (MI/fast)	Danish, Belgian, Italian
	"Dimer"	Welsh, Swedish
Negroid	Cayemite	Negro (Haiti)
	Uinba	New Guinea, indigenes
	New Guinea (Reading)	New Guinea, indigenes
	"Dimer"	Negro (USA)
Mongoloid	Gombak (Paris)	Indonesian
	Kashmir (Afghanistan)	Pushtoon (Afghanistan)
	Pushtoon	Pushtoon (Afghanistan)
	Mexico	Indians of South Western USA and Mexico
	Medán	Malayan aborogine
	Máku	Yanomamö (Venezuela), Makiritare (Brazil)
	Makiritare-3	Makiritare (Brazil)
	Naskapi	North American Indian
	Makiritare	Warao, Makiritare (Venezuela), Trio, Wajana (Surinam)

Race	Variant	Population
	Yanomama	Yanomanö (Venezuela)
Unknown	B	Negroid extraction (USA)
	Santa Ana	Caucasoid or Mongoloid (Mexico)
	Belem I Belem II Belem III	Trihybrid group of Caucasoid, Negroid and Mongoloid mixture (Brazil)

Albumin variants of Amerindian origin are Albumins Naskapi, Mexico, Makiritare, Máku, Yanomama and Makiritare (Warao). Another two variants, Santa Ana and Belem I, may be of Indian origin[303]. The overall pattern shows polymorphism for albumin Naskapi in north North America (gene frequency 0.14[181]), lower gene frequency for Albumin Mexico in south North America (gene frequency 0.03[183]), and even lower gene frequency (0.014) for Albumin Makiritare (Warao)[303]. It would be interesting to find the precise function of this polymorphism although their finding might suggest a north to south migration from Asia[89,303].

The slow Albumin B (population frequency 1: 1,000-10,000) and fast Albumin Gent, are two of the most frequently found variants in Europeans[303]. It is interesting that albumin B and its subtypes[197] should be found so frequently in Europeans of numerous diverse backgrounds and it is difficult to imagine that it arose from a common founder allele, or identical random mutation.

Some of the other variants in Europeans are the only case of variant recorded in Poland (Gdansk), probably of Jewish origin[186] an original, spontaneous

mutation found in Austria[125], numerous cases in Italy and their immigrants to USA[217], about 14 Spanish[130,131,161,194,288] and at least a dozen cases in Britain[64,67,87,202,279].

In Japan, the population frequency of bisalbuminemia is higher (population frequency 1:400) in Hiroshima and Nagasaki (mutations most probably catalysed by radioactivity) compared to the general frequency of 1:6,000 of which about twenty-two cases are reported[79,94,188,305]. Some variants found in the India subcontinent are AlbuminsKashmir (Afghanistan) and Pushtoon[276,298]. Some of these variants are described in the following sections and are subdivided into fast and slow monomeric variants and dimeric variants.

1.6.2.8 Slow Monomeric Albumins

1.6.2.8.1 Albumin B

Albumin B appears to be a broad classification of the widely distributed variant found in Europeans with a population frequency of 1:1,000-10,000[297]. Heterozygotes of this variant are found throughout Europe and their migrants in Argentina and the USA[50,73,160,312].

One of the earliest B albumins was found in an American family of German descent and was named for their surname 'Oliphant'. It was originally thought to be a 'private' variant but was later found in 22 families, and is an identical allomorph of Albumin Ann Arbor found in a family originating from Denmark and Albumin B[311].

Albumin Gainesville, found in an American of Irish descent[160] was classed as B' by Ott[199]. Its site of mutation is located between residues 1-122[157].

Albumin Paris[235] which migrates almost identically to the fast-moving albumin was subclassed as B" [199]. A homozygous albumin B, called Albumin B Schönaich, was found in Stuttgart[198].

About 25-30 slow type albumin B have been discovered in France[22,81] including a homozygote whose parents are relatives[174], two families in investigation of 10,000 sera in England[64], and one case in Norway[74].

The majority of albumin B are found to be immunochemically identical to albumin A but a supposedly structurally different albumin B was detected by immunoprecipitation but not by immunoelectrophoresis[190].

The diminished anionic character of albumin B on electrophoresis is due to replacement of glutamic acid-570 by basic lysine[101,311]. Gitlin and Gitlin[101] proposed that since the C-terminal segment is unnecessary for the stability of the tertiary structure, it is more vulnerable to mutation.

Study of the turnover rate and distribution of albumin A and B by *in vitro* labelling [^{131}I]-normal and [^{125}I]-slow albumins showed no significant differences[29]. Albumin B had a similar affinity in bromophenol blue binding but bound thyroxine more tightly than albumin A. Reversed albumin A/globulin ratio was noticed on the slow variant of Creason and Creason[66].

1.6.2.8.2 Albumins Kashmir and Afghanistan

Albumin Kashmir was originally found in a Moslem family living in London but originating from Kashmir[277]. Although it is one of many types of variants found in the Indian subcontinents, with a frequency of 1:500 among 1,500

Asian Indians, it has also been found in other populations in the Indo-Dravidian group[297]. In studying bisalbuminemia in Hawaian-Japanese[94] it is argued that Indians are anthropologically classed as Caucasians and, therefore, this variant should not be qualified as a case of Asian albumin polymorphism.

This variant binds slightly more bromocresol green (52%), has impaired binding of HABA, and binds more bromophenol blue on filter paper (reversed on cellulose acetate) than albumin A[277].

Albumin Afghanistan was found in 10 individuals of a 251 strong Pushtoon tribe that had originated from Afghanistan near Kashmir[300]. It is similar to Albumin Kashmir when tested by 3 pH starch gel systems of Weitkamp.

1.6.2.8.3 Albumin Mexico

The distribution of this polymorphic variant is wide, from central Mexico (especially amongst the Uto-Aztecan language group) to as far south as northern Gautemala and the linguistically and culturally-related Pima and the Papago and Zuni Indians of south western USA, who live contiguously to the Navajo and Apache[297]. The Apache may have acquired this variant through raids in Pima and Spanish villages in the 17th and 18th Centuries[254]. It was also found in a trihybrid Brazilian population[303]. Both Albumin Naskapi and Mexico are found in the Navajo, Apache and Hopi in high frequencies. All homozygotes of these variants are healthy.

However, it has not been detected, so far, in

northern North American Indians, Eskimos, two Peruvian Indian tribes, Mayan, and a limited number of half castes.

Mexican sera which had been stored for five years in deep freeze migrated between albumins A and B on electrophoresis at pH 8.6[183]. Two species of Albumin Mexico named Albumin Mexico I and II, can be distinguished by comparisons of peptide maps.

The latter arise from at least a 550-glycine to aspartic acid substitution. This substitution of helix-indifferent aspartic acid by strong helix breaker, glycine, in the α-helical region is sufficient to cause a gap of about 4 residues in the helix[90] when predicted using the rules of Chou and Fasman[59]. This mutation is also in Domain III, the site of long chain fatty acid binding and is also one of the primary binding sites for medium chain fatty acids[169] (Section 1.5.3.4).

The variant binds haemoglobin and thyroxine equally well but dyes[181] and warfarin[308] are bound less readily than albumin A. The mean number of warfarin binding sites per normal albumin molecule is 1.23 $\pm$ 1.7 and the mean association constant is 1.62×10^5 M^{-1}, but these values are diminished to 1.40 $\pm$ 0.18 and 1.26×10^5 M^{-1} respectively in Al^A/Al^{Me}. The clinical implication of decreased binding is an increase in pharmacologically active free warfarin in the circulation leading to an altered pharmacological response.

1.6.2.8.4 Albumins Christchurch and Lille

Albumin Christchurch contains a propeptide sequence of arg-gly-val-phe-arg-gln[40,41] and Albumin Lille, arg-gly-

val-phe-his-arg[1] instead of the normal human propeptide sequence arg-gly-val-phe-arg-arg[72].

These circulating proalbumins are in the same proportion to albumin A and can be easily proven to be proalbumins by cleavage of the propeptide by mild tryptic treatment.

The existence of these circulating variant proalbumins gives an insight into the *in vitro* recognition factor in propeptide cleavage. Paired basic residues at the C-terminal of the propeptide appear to be a prerequisite for *in vivo* cleavage but the finding that the chicken propeptide only contains one arginine, but is nevertheless cleaved, suggests instead the requirement of a non-specific tryptic-like protease[78].

Impaired Cu(II)binding due to lack of high affinity binding site was found in Albumin Christchurch[41]. A similar aspect in Albumin Lille, plus defective Ni(II)binding, can be expected in both variants (Section 1.5.4.2).

Albumin Christchurch binds stearate more strongly and myristate less strongly than albumin A. Both albumins bind palmitate, ANS and bromocresol green equally well. When compared to control normal albumins, this variant and albumin A contain more myristate and sterate but less oleate and linoleate[41].

1.6.2.8.5 Albumin Xavante

This variant, originally found in the Xavante Indians of the Brazilian Mato Grasso, is one of four (besides Yanomama, Yanomama-2 and Makiritare-3) variants so far found exclusively in American Indians[302].

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The electrophoretic mobility of Xavante and Albumin Uinba are indistinguishable from normal albumin the pH 6.0 and 6.9 buffer systems but can be identified from Albumin Pushtoon in the latter system.

1.6.2.8.6 Albumin Yanomama-2

This polymorphic variant was found in 47 individuals from three Yanomama villages in northern Brazil, which included one homozygote[272].

Its electrophoretic mobility is similar to albumin A at pH 5.0 and 6.0 but is only slightly different at pH 6.9[302] and is distinguishable from Albumin Máku[303].

1.6.2.8.7 Albumin RS-1

This comes from East African and is Indo-European in origin. It is similar to SO/BS on electrophoretic cellulose acetate at pH 9.25[216]; however they can be discriminated on pH 5.0 and 6.0 starch gel systems. Unfortunately in this system, it is similar to albumin Pollibauer but can be shown to be different in the pH 6.9 system where it is slightly faster[302].

1.6.2.8.8 Albumins Gombak and Paris

Both albumins, originating from Malaysia[166] and France[303] are similar and are unstable in that they will merge into albumin A on prolonged storage. This may be because this variant is actually albumin A with charged ligands bound to it and these become detached during freezing.

1.6.2.8.9 Albumin Adana

So far, this variant is found to be restricted to the district of Adana in South Eastern Turkey, unlike Albumin Mersin which is frequently found amongst the

Eti Turks living in the same region. The reason may be that these two groups of Eti Turks are ethnically distinct and these albumin variants arise from inbreeding or genetic drift within local communities.

Albumin Adana can be distinguished from albumin B by PAGE and the site of mutation is located between residues 549 and 585[89].

1.6.2.8.10 Albumin Cayemite

This variant was found in a Negro mother and son as a result of investigations in Savannah/Georgia USA and Grand Cayemite Island/Haiti. Although two distinct bands are manifested in all three pH systems, the best resolution was in acid pH[299].

1.6.2.8.11 Albumin Cartoga

Eighteen individuals out of 1,400 sera in Cartoga/Costa Rica carried this anomaly. At pH 8.6, its electrophoretic mobility was similar to Albumin Santa Ana and inbetween Albumins Mexico and Gainesville. At acid pH (5.0 and 5.4) it is similar to Albumins Uinba, Mexico, Cayemite and B[161].

1.6.2.8.12 Albumin Santa Ana

This variant was found in a Mexican family living in California and is suspected to be Indian in origin. At pH 5 and 6, its electrophoretic mobility is alike Albumins Cayemite and B but not Albumin Mexico[152].

1.6.2.8.13 Albumin Pushtoon

This variant with a gene frequency of 1-2% was found during a multidisciplinary study in a Pushtoon village which had migrated from a region in Afghanistan.

It is separable from normal albumin on electrophoresis using the three pH systems, pH 5.0, 6.0 and 6.9 but not at pH 8.6[300].

1.6.2.9 Fast Monomeric Albumins

1.6.2.9.1 Albumin Reading and New Guinea

Albumin Reading, of Welsh ancestry, was found in a 75 year old male and his two female near relatives[279]. Like most fast variants, electrophoretic separation was better at pH 5.4 than pH 8.6[242]. It is identical to Albumin New Guinea which is found in a totally different and separate population, in the pH 5.0 and 6.0 gel buffer system of Weitkamp[297].

The authors found that bromophenol blue bound only to Albumin Reading but not albumin A. Binding to the latter was achieved only at the same level at which globulins were also stained. Another dye, ANS (11) travelled with albumin on filter paper but on cellulose acetate electrophoresis the dye became detached probably due to differential affinities for the media. Albumin A bound more thyroxine than Albumin Reading.

Albumin New Guinea was found in about thirty individuals in New Guinea and surrounding island[169,178,299]. Weitkamp[302] quotes the gene frequency as 0.017.

1.6.2.9.2 Albumin Gent

This variant, previously named Albumin Fast or Albumin Very Fast, was found in a family of seven. A further 17 individuals were later found[367].

Similar to most fast variants, bromophenol blue was bound with much higher affinity.

1.6.2.9.3 Albumins Naskapi and Mersin

This variant[182] is one of the three polymorphic variants, besides Albumins Mexico and Makiritare, to be of Amerindian origin.

The dispersion of this variant is from the Canadian and North American Indians [Naskapi, Montagnais, Sioux, Athabascan (Navajo, Apache) and Shoshonean (Hopi)] to Eskimos from Alaska and Labrador. Indians from the middle and south USA, South Americans nor Africans, Asians, Oceonics and Caucasians have yet to be found with this variant. The discovery of this variant in so many tribes have proven anthropological links, for example, between the English-speaking, Anglican Naskapis and the French-speaking, Roman Catholic Montagnais, both of whom are Algonkian-speaking tribes. One plausible explanation for its polymorphism is inbreeding through several generations.

The genetic distribution of Albumin Naskapi on Table 1.3 shows the heterozygotic frequency to be 8.48% and the homozygous frequency as 0.36%.

This variant segretates genetically with Gc-1 phenotypes[140] and the site of mutation is located between residues 330 and 446[89]. This is on the β-turns of albumin (Fig. 1.4) of Domain II, in between the high affinity binding sites of bilirubin (Section 1.5.2.3) and on one of secondary binding sites for medium chain fatty acids[164]. It is not known if bindingsof these ligands are impaired.

An identical allomorph is albumin Mersin found in high frequency amonst the Eti Turks of south eastern Turkey. The heterozygote frequency is 8.8% in Mersin

TABLE 1.3 The genetic distribution of albumin Naskapi

Tribe	Number	$Al^{Na/Na}$	$Al^{A/Na}$	$Al^{A/A}$
Naskapi (Canada)	203	1	54	148
Montagnais (Canada)	128	2	19	207
Sioux (N. America)	160	0	2	158
Athabashan (USA & Canada)	230	1	11	218
Eskimos (Labrador)	124	0	3	121
Eskimos (Canada & USA)	262	0	4	258
Total	1,107	4	93	1,010
% of Total	100	0.36	8.48	91.23

Adapted from Blumberg[35].

and 9.7% in Tarsus. This discovery of its similarities to albumin Naskapi is anthropologically interesting because it strongly suggests the ancestry of these Indians to be from Mongoloid populations in Central and/or Eastern Asia[89].

Albumin Naskapi binds more bromophenol blue[181] and thyroxine[34] more tighly than albumin A. Both albumins bind linolenic acid, linoleic acid[55] and haemoglobin equally well[181]. Only albumin A binds riboflavin and warfarin[55]. Reaction of penicillin with heterozygous Albumin Naskapi produces four bands[13].

1.6.2.9.4 Albumin Máku

This widely distributed variant is found in tribes living in the Southern borders of Venezuela and Brazil[301]. The original sighting was in a captured Máku woman and her three near relatives living among the Yanomamö tribe of Southern Venezuela. It was later found

in two other individuals in adjacent north central Brazil (Makiritare-2) and in the trihybrid population of Northern Brazil (Belem III)[303].

The migration of this variant is faster than Albumin Naskapi at shorter (5-6 hr.) length of electrophoresis, but this is reversed at longer (19 hr+) running times[30] probably because Albumin Naskapi is able to bind more charged ions during the longer running time to increase its relative mobility.

1.6.2.9.5 Albumin Syracuse

This was found in a family of Swiss German descent. Minimal separation was obtained on electrophoresis at pH 8.6 but the variant separated well on cellulose acetate at pH 5.4[242].

1.6.2.10 Dimeric Albumins

1.6.2.10.1 Warao

This dimer was found on two natives of British Guyana[11] and in five familial members of a Warao tribe living in the Orinoco delta[12]. This tribe is thought to originate from the Andes and blood group-typing detected Karib or Arawak Indian phenotypes.

This dimer constitutes 30% of the total albumin content and can be separated easily into monomers by heating at 56°C for 30 min. Susceptibility to dimerisation may characterise this variant.

Its electrophoretic mobility is close to α_1-globulin on cellulose acetate and close to the fast-moving α_2-globulin in starch gel. It can be distinguished from European dimers in the pH 6.0, but not in the 5.0 and 6.9 starch gel system of Weitkamp.

1.6.2.10.2 Albumin Makiritare

This dimer is found amongst the Makiritare and the Piaroa tribes of Venezuela, and the Trio and Wajana of Surinam. The average gene frequency is 0.014, arising from a total of 41 affected individuals, out of 1,461 members of the four tribes: Makiritare, Warao, Trio and Wajana[303]. This hereditary dominant variant is also thought to be alike Albumin Warao found in the Warao of Venezuela and Wapishana of Guyana[297].

1.6.2.10.3 Albumin Yanomama

This variant originates from the Yanomamö Indians living in South Venezuela and Northern Brazil. They separate on electrophoresis at pH 5.0 and 6.9 showing a slight separation of monomers from dimers[298].

1.6.2.10.4 Albumin dimers in Sweden, USA and Wales

Five carriers were found amongst some orthopaedic patients in a Swedish clinic although a connection between this hereditary anomoly with orthopaedics is unproven. A large number of affected individuals were found in nine of the 24 relatives of a proband, including a brother who was homozygous.

The albumin dimer found in a healthy American Negro carrier and 6 members of his family of nine[137] could be dissociated into monomers after reduction and alkylation[136].

The electrophoretic mobility of the Welsh dimer, which was the first reported case of hereditary dimers[91] is similar to a dimer found in the USA, except that they differed in binding of alkaline haematin and haemoglobin. There were six carriers in a family of

11 spanning three generations.

1.6.3 Non-hereditary transient bisalbuminemia

1.6.3.1 Introduction

Transient bisalbuminemia is a rarer phenomenon than hereditary bisalbuminemia with about 50 reported cases from 1948 to 1979. It can be easily recognised as it appears in lesser quantities than normal albumin during acute illness as a fast (anodic) band adjacent to the normal allotype. It usually disappears before complete recovery from illness is secured. The fast band can be induced by ligand binding or by pancreatic diseases.

Unlike hereditary bisalbuminemia, the drug- and enzyme-induced forms are not usually clear distinct bands *in vivo* or *in vitro*, except within certain limits[215]. Both albumins are usually immunochemically similar and investigation of familial albumin excludes the hereditary factor.

The initial reports of transient persistant double albumins were by Glatthaar[103], Sandor[234], Brodenstein and Rau[42] and is reviewed by Tárnoky[274] and Porta[215].

1.6.3.2 By Pancreatic Diseases

A fast albumin component was observed in a 47 year old woman with subacute pancreatitis with hyperamylasemia. The concentration of the fast component was later recognised to be directly proportional to the serum amylase level. It constituted less than 50% of the total serum albumin, even when the serum amylase level was at 3,000 Somogyi level[245].

However, with the report of a case of bisalbuminemia associated with chronic pancreatitis which was indifferent to the level of amylase was reported, Lamotte-Barillon[155] suggested structural modification of albumin A by trypsin.

Pancreatic enzymes were later shown to cause formation of a more anodic component by limited proteolysis of albumin A in a clinical case of pancreatitis with hyperamylasemia. The same effect could be reproduced *in vitro* by the limited action of chymotrypsin with carboxypeptidase A and B and elastasis-carboxypeptidase A and B on albumin A. Trypsin acts merely to activate the zymogens of the above enzymes[68].

The action of chymotrypsin or elastase followed by carboxypeptidase A and B was to remove a C-terminal fragment, therefore, accounting for a fast mobility albumin.

1.6.3.3 Binding of Ligands

Albumin A can be transformed into an electrophoretically distinct rapid molecule by binding anionic ligands. This transformation may be a balance in favour of anions bound to rather than those that bind reversibly through weak associations.

Arvan[13] noted that a small fraction containing penicillin G bound to albumin A was electrophoretically faster than albumin A because of the increased anionic character contributed by the carboxylic acids on the drug. They showed that heavy prescription of cephalothin and penicillin G (20 to 60 million units/day) but not

chlorophenicol nor streptomycin could induce this transformation. *In vitro*, cephalosporins can induce bisalbuminemia (Hutchinson, private communication).

Disproportionate ratios of albumins could be induced *in vitro* by adding increasing quantities of penicillin G (124 to 22,700 IU/ml serum), the concentration of fast form being proportional to quantities of drug.

Inactivation of penicillin by an equal quantity of penicillinase at 25°C did not induce formation of the fast form since an opened β-lactam ring was functionally useless as shown on Scheme 1.2.

Scheme 1.2 Cleavage of penicillin G by penicillinase

This observation was used as confirmation of mechanism of penicillin binding by Bungaard[53] (Section 1.5.5.2). Guibaud[110] related the cause of transient bisalbuminemia to high dosage of β-lactam type antibiotics.

Porta[214] standardised the *in vitro* conditions for progressive induction of penicillin-albumin from albumin A by incubation 500-30,000 units of penicillin/ml serum at 37°C for 3 hours. Equal albumin bands were formed at 10,000 units. The same effect could be obtained by increasing the penicillin concentration but decreasing the incubation time[212]. As expected, normal bands

predominate at low penicillin levels but the fast albumin is formed at increasing penicillin levels. Penicillinase (bacterial β-lactamase) at 256-500 units/1,000 units of penicillin completely hindered formation of double bands.

Only two or three moles of penicillin were originally thought to be bound per mole of albumin[83] but the more sensitive and specific method, radioimmune assay, uncovered 5-10 more penicillyl groups bound to the fast component than the normal[159]. Cleavage of the fast component by cathepsin D increased the concentration of penicillyl groups 50 fold, but pronase or subtilisin further increased this value by 100-200 fold.

Penicillin binding does not impair binding of bromophenol blue to albumin[288]. Tárnoky and Porta[280] showed that bisalbumins induced *in vitro* by penicillin had less affinity for bromocresol green and bilirubin than normal sera or hereditary variants. Reduced dye binding capacity could be achieved by the action of chymotrypsin or carboxypeptidase A and B.

1.6.4 Analalbuminemia

This is the near absence or complete absence of albumin in an individual, and its existence casts doubts on the vital requirement and obligate functions of albumin discussed in Section 1.1. It may be inherited as an autosomal recessive gene and can be found in offspring of consanguineous unions[21].

Other proteins, such as haptoglobins, α_1-antitrypsin, ceruloplasmin, α_2-macroglobulins, Ig G[109], transferrin, β-lipoprotein and fibrinogen[65,69,109,291]

are present in elevated amount probably to substitute the functions of albumin.

Christen and Franglen[61] suggested the possibility of two forms of analalbuminemia. The most probable reasons are that albumin is absent or made in minute quantities. Alternatively, the albumin may be produced in say, a modified form which also has a tendency to form complexes with other proteins, and so escape detection. The latter possibility was not confirmed.

In analalbuminemia the half life of intravenously-injected labelled albumin is prolonged to 120 days, instead of the normal 20 days[101]. Undeveloped rough endoplasmic reticulum contributes to the decreased rate of albumin synthesis although a minimal amount of albumin is produced by a few healthy cells[141].

Albumin levels are difficult to estimate at such low levels but figures of 16-240 mg/l are measured by sensitive immunologic methods[21,195] compared to 42 g/l in a normal individual.

Despite such low albumin levels, most symptoms are mild. Colloidal pressure which is decreased to a third or half normal value is compensated by a slight decrease in blood pressure and low capillary pressure. Most patients have elevated serum, cholesterol and phospholipid but normal triglyceride levels. Transport disorder is indicated by an increase in elimination of intravenously injected Congo Red and Evan Blue.

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CHAPTER II

2.1 INTRODUCTION

In 1980 a young Punjabi male was hospitalised for ulcerative colitis in Warwick Hospital. The cellulose acetate electrophoresis pattern of his serum proteins showed two distinct and separate forms of albumins (Fig. 2.1). The rare, extra albumin was of slower electrophoretic mobility than normal albumin, and appeared to be equal in concentration to normal albumin.

(i) (ii)

Fig. 2.1 Cellulose acetate electrophoresis pattern (i) Mundey's serum, (ii) normal serum.

A later investigation of the familial albumin established hereditary bisalbuminemia. The pedigrees in Mundey family with bisalbuminemia is shown on Fig. 2.2 and the electrophoretic pattern of their serum on poly-

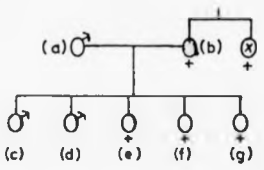

Fig. 2.2 Genealogical tree of the Mundey family with bisalbuminemia.
O = positive, O = negative, ⊗ = not tested
The age of the offspring decreases from left to right.
The subject is the younger of the two sons.

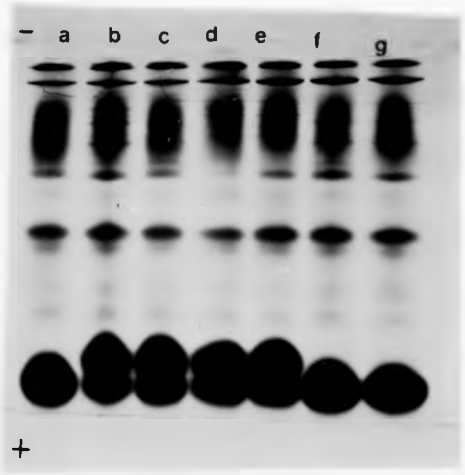

Fig. 2.3 Analysis of the serum of the Mundey family on disc-PAGE[6].

acrylamide gel[6] is shown on Fig. 2.3. So far, this anomalous albumin is not associated with any known diseases, except incidentally. We propose to call this slow alloalbumin "Albumin Mundey" for their surname.

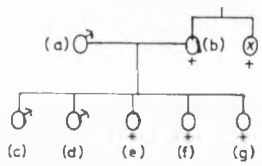

Fig. 2.2 Genealogical tree of the Mundey family with bisalbuminemia.
○ = positive, O = negative, ⊠ = not tested
The age of the offspring decreases from left to right.
The subject is the younger of the two sons.

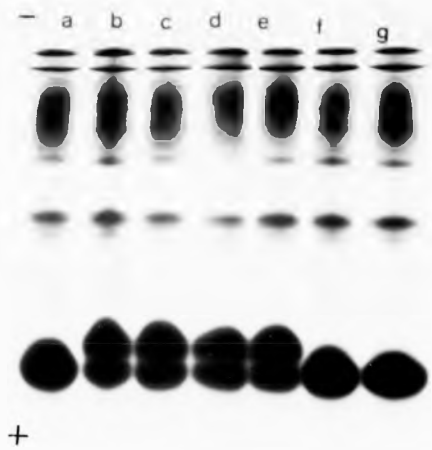

Fig. 2.3 Analysis of the serum of the Mundey family on disc-PAGE[6].

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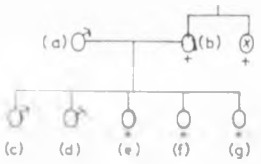

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The age of the offspring decreases from left to right.
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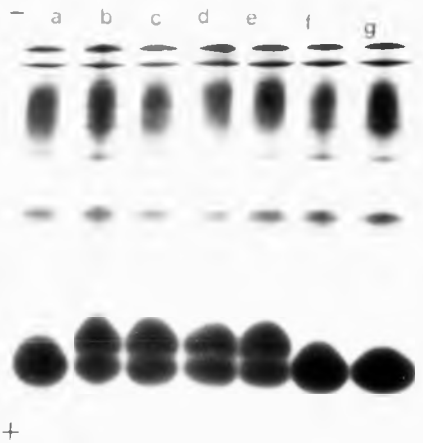

Fig. 2.3 Analysis of the serum of the Mundey family on disc-PAGE[6].

acrylamide gel[6] is shown on Fig. 2.3. So far, this anomalous albumin is not associated with any known diseases, except incidentally. We propose to call this slow alloalbumin "Albumin Mundey" for their surname.

2.2 AIMS OF THIS PROJECT

Hereditary bisalbuminemia is the rare occurrence of amino acid mutation(s) in the primary sequence of normal albumin to produce a second albumin of either slower or faster electrophoretic mobility in the serum (Section 1.6.2).

The main objective of this research was to identify the mutant amino acids and to locate their positions on the primary structure of Albumin Mundey.

2.3 EXPERIMENTAL STAGES

This research was performed in five successive stages. The first stage was the characterisation of Albumin Mundey using well-known methods of classification (Sections1.6.2.5 and 1.6.2.6). The information gathered at this important initial stage was used to distinguish it from other well-established Indian variants, Albumins Kashmir[16], Hussain, Birmingham[3] and Vancouver[8].

The second stage was the isolation of Albumin Mundey from normal albumin in an absolutely pure and homogenous state. This condition was stringent so that any unusual peptides observed in the comparison of the peptide maps of Albumin Mundey and the control (normal HSA) could be attributed solely to the mutant peptides.

The elements of the third stage, sequence determination, involved the fragmentation of Albumin Mundey into fairly large and accountable fragments. To achieve

this, the sites of cleavage were specifically aimed at the amino acids, rare in the primary sequence of HSA. These amino acid residues are the six methionines and the single tryptophan.

The fourth stage was the detection of the mutant peptides by peptide mapping. Using this technique the identities and locations of the peptides on albumin were identified.

The final stage was the identification of the mutant amino acids by finger printing methods. To achieve this, the mutant fragments were isolated and then cleaved further into smaller peptides or hydrolysed completely into a pool of amino acids.

These five successive stages are described in the following chapters.

CHAPTER III

STAGE 1: CHARACTERISATION OF ALBUMIN MUNDEY

3.1 INTRODUCTION

The characteristics of Albumin Mundey that were determined are listed.

(i) Electrophoretic mobility[17] and the quantitative ratio of albumins in serum on six electrophoretic media.

(ii) Ligand binding capacities[10] of albumins in serum as detected by the same six electrophoretic media.

(iii) Quantification of ligands bound to albumins as detected on cellulose acetate.

(iv) Proportion of Albumin Mundey to normal albumin in serum.

(v) Total protein concentration in serum[7].

(vi) Total albumin concentration in serum[9].

(vii) Thermal stability[2,10].

(viii) Molecular weight[11,19] determination, including the possibility that it may be a dimeric variant[12].

(ix) Detection of N- or C-terminal peptide[1,4].

(x) Immunochemical character[13].

(xi) Isoelectric point.

(xii) Genetic linkage to group specific component (Gc-component).

Dr. A. L. Tárnoky at the Royal Berkshire Hospital kindly classified Albumin Mundey for us by

Table 3.1 Standard Conditions for Determining the Electrophoretic Mobility of Albumin Variants

Media	Electrophoresis buffer	Electrophoresis Condition		
		Current (mA)	Voltage (v)	Time
(a) Cellulose acetate (Shandon Cellagram)	Barbitone (pH 8.6, 0.1 M)	7.5		1 h. 20 min.
(b) Cellulose acetate (Helena Titan III Zip Zone)	Barbitone pH 8.8, 0.05 M		110	35 min.
(c) Filter paper (Whatman 3 mm 36 x 5 cm)	Barbitone (pH 8.6, 0.05 M)	3.5		7 h. 30 min.
(d) Agar (1%)	Barbitone (pH 8.6, 0.05 M)		200	1 h. 30 min.
(e) Agarose (1%) (Corning ACI Electrophoresis System)	Barbitone (pH 8.6, 0.1 M)		90	45 min.
(f) disc-PAGE[15] (7%, pH 8.9)	*Tris* (0.05 M)/ glycine (0.38 M) pH 8.3		2-3 mA/gel rod	

determining factors in Section 3.1(i, ii, and xii). A comparison of its electrophoretic mobilities and ligand binding capacities was made with Albumins Kashmir (Section 1.6.2.8.2) and Hussain on the six electrophoretic media. Data on two other albumin variants of Indian origin Albumins Birmingham[3] and Vancouver[8] were also used for comparisons. Albumin Birmingham and this variant both have ancestral origins in the Punjab, India.

Chromatographically pure normal albumin was prepared as described in Chapter IV.

3.2 EXPERIMENTAL

3.2.1 Electrophoretic mobility[17]

Table 3.1 summarises the electrophoretic conditions used to determine the electrophoretic mobility on six standard media.

3.2.2 Ligand-binding capacity[10]

Serum (100 µl) was incubated with 50 µl of 0.05% solutions of bromophenol blue, Ponceau S, HABA, bromocresol green, bilirubin and Congo Red for 10 min. at 37°C. The molar ratio of albumin:dye was about 2:1 except for HABA where this ratio was 1:2. The albumin-ligand complexes were then separated on the six electrophoretic media. Qualitative estimation of the ligands bound or the positions of the unbound ligands

were noted before staining. All, except the paper electrophoretogram, were stained in Ponceau S (0.2% w/v) in TCA (3% w/v). The paper chromatogram was stained in bromophenol blue (1% w/v) in ethanol (95%). The position of the albumins were again noted after destaining.

3.2.3 Quantification of bound ligands on cellulose acetate

The ligands were bound to the albumins in Mundey's serum using the procedure in Section 3.2.2, except that the solution of HABA was at pH 6.2 and bromocresol green at pH 3.9. Electrophoresis on cellulose acetate was in barbital buffer (pH 8.6, 0.05 M) at 12 V/cm for 1 h. 20 min.

To quantify the ligand bound, the cellulose acetate strip was cut in half parallel to the direction of electrophoresis. One half was used to observe the relative quantity of ligand bound by albumins or the positions of the ligands before staining in Ponceau S. The albumin bands in the second half (located by the stained first half) were excised and the albumin-ligand complexes eluted from each excised band with equal volumes of ethanol (2 ml), except for the bilirubin-albumin complex which was eluted in chloroform (2 ml). The UV absorbances of the eluted bromophenol blue, Congo Red, bromocresol green and bilirubin was measured at 590, 488, 630 and 453 nm respectively.

3.2.4 Proportion of albumins in serum

The albumin bands separated by cellulose acetate

electrophoresis were initially located by a Ponceau S stained guide strip. The individual bands were excised and then the albumins eluted with NaOH (1 M, 2 ml). The eluates were then quantified by the UV absorbance at 280 nm.

3.2.5 Estimation of total protein in serum[7]

The total protein content was determined by the Biuret method. Chromatographically pure normal HSA was used to construct a standard curve.

3.2.6 Estimation of total albumins in serum[9]

Serum (10 µl) was added to the dye solution (2 ml). This was followed by very rapid mixing and almost immediate measurement of the absorbance at 628 nm by UV spectrometry.

The dye solution consisted of bromocresol green (0.15 mmol) in succinate buffer (75 mmol) and Brij-35 surfactant (4.0 ml, 300 g/l), pH 4.20. Water (10 µl) was substituted for the serum in the blank. Chromatographically pure HSA was used to construct a standard curve.

3.2.7 Thermal stability

The sera were subjected to heating at 37°C and then freezing at -20°C ten times[10] as well as to heating at 56°C for 30 min. and 2 h[2]. The thermal stability was

then determined by observation of the mobility of Albumin Mundey against the control variant sera on cellulose acetate.

3.2.8 Molecular weight determination

3.2.8.1 By disc-SDS-PAGE

50 µg of double albumins were loaded per gel (7.0 x 0.6 cm i.d.) and electrophoresis carried out by SDS-PAGE[19] (7.5%) in sodium phosphate (pH 7.2, 0.2 M) system containing SDS (0.2%) at 6 mA/gel. The molecular weight marker used was chromatographically pure normal HSA.

The molecular weight was also determined by disc-SDS-PAGE[11] consisting of a stacking gel (4%) in *Tris*-HCl (pH 8.8, 0.375 M). Both gel buffers contained SDS (0.1%).

After electrophoresis the proteins were fixed and SDS removed in isopropanol/acetic acid (25:1 v/v). Staining was in Coomassie Blue R-250 (0.1%) and destaining was in methanol/acetic acid/water at 40^{o}C (5:2:13 v/v/v) containing $CuSO_4$ (0.5%) to decrease the blue background[14].

3.2.8.2 By Gradient SDS-PAGE[12]

The possibility that Albumin Mundey may be a dimeric variant was investigated by 'sieving' this protein on a gradient gel (5-22.5%) containing *Tris*-HCl (0.375 M, pH 8.8).

The stacking gel (4.75%) contained *Tris*-HCl (0.125 M, pH 6.8). Both gel buffers contained SDS (0.1%). Electrophoresis was at 125 V. The albumins were fixed in

sulphosalicyclic acid (10%), stained in Coomassie Blue R-25 (0.1%) in destaining solution, methanol/acetic acid/water (25:10:65 v/v/v) followed by destaining. Chromatographically pure normal HSA was used as a molecular weight marker.

3.2.9 Detection of N- or C-terminal peptide

The possibility that Albumin Mundey may have an N- or C-terminal peptide, whose ionic charge may account for its slow electrophoretic mobility, was investigated by reaction with mild trypsin under optimised conditions[1,4].

3.2.9.1 With Serum

Serum (10 µl) and TPCK-trypsin (5 µl, 0.1% w/v) in ammonium carbonate buffer (pH 8.0, 0.1 M) were incubated for 2 h at 37°C. Trypsin was not added to the control sera. The electrophoretic mobilities of these two samples were compared by cellulose acetate electrophoresis.

3.2.9.2 With Albumin Mundey

Pure Albumin Mundey (2% w/v) dissolved in ammonium carbonate buffer (pH 8.0, 0.1 M) was added to TPCK-trypsin solution (2 µl). The ratio of trypsin to protein was 0.2% (w/v). Incubation and detection procedures were as for whole serum. The control was Albumin Mundey without any added trypsin.

3.2.10 Immunochemical character

The immunochemical character of Albumin Mundey

(isolated and in serum) was determined by immunoprecipitation[12] with goat antiserum monospecific for normal HSA. The binding capacity of the antigen was initially determined with serial dilutions of normal HSA.

3.2.10.1 Preparation of Agar Gel

A solution of agar (electrophoretic grade, 1% w/v, 20 ml) in sodium phosphate buffer (pH 7.1, 0.25 M) and sodium azide (1% w/v) was poured into sterile petri dishes. Underrunning of sera and antisera was prevented by smearing the petri dish with a thin layer of grease and then layering a thin film of agar powder before the addition of the agar solution. Sample wells (0.5 cm diameter) were cut with a mechanical cutter and the contents removed by suction.

3.2.10.2 Determination of Antigenic Binding Capacity

Antiserum (200 µl, 4.0 mg/ml) was mixed with sodium phosphate buffer (100 µl, pH 7.2, 0.05 M) in the control sample well. Serial dilution of chromatographically pure normal HSA were added to the peripheral sample wells such that the antigen/antibody ratio was 1:1, 1:2 and 1:4 (w/v). The total volume in each sample well was 300 µl solution. The best binding capacity was found to be an antigen/antibody ratio of 1:1 (w/w). The immunoprecipitation reaction was allowed to occur in a constant-moisture chamber for 24 h at 37^{o}C. Once the precipitation lines were obtained, the bands were fixed in 20% methanol/ saline (1:1 v/v).

3.2.10.3 Immunoprecipitation of Albumin Mundey

Albumin Mundey (0.4 mg) in sodium phosphate (300 µl, pH 7.2, 0.05 M) was reacted against antiserum

(200 µl) in the same phosphate buffer (100 µl). The control was chromatographically pure HSA (0.4 mg).

3.2.10.4 Immunoprecipitation of Double Albumins in Serum

Serum (10 µl) in sodium phosphate buffer (pH 7.2, 0.05 M) was reacted against antiserum (200 µl) with pure normal HSA (0.4 mg) as control.

3.2.11 Determination of isoelectric point of Albumin Mundey

The isoelectric point of Albumin Mundey was determined by electrofocusing in thin-layer and rod polyacrylamide gel. An approximation of this value was initially obtained by electrofocusing in the wide pH range (pH 3-10) followed by analytical determinations in the narrow pH range (pH 4-6.5).

3.2.11.1 Analytical Electrofocusing in Thin-Layer Polyacrylamide Gel

Electrofocusing in thin-layer polyacrylamide gel (1 mM, T = 5%, C = 3%) was at 30 W constant power at 10°C for 2.5 h on LKB Multiphor 2117. The isoelectric points of albumins [defatted[5] and native (not defatted)] were determined.

3.2.11.2 Preparation of Electrofocusing Gel

A glass plate (25 x 12.5 cm) was pretreated with silane A-174 to ensure adhesion of the gel to the plate during prolonged staining and destaining procedures. A plastic sheet (Gel Bond) was attached to the other plate to prevent adhesion of gel. The gels, containing Ampholyte (pH 3-10) or Pharmalyte (pH 4-6.5), were cast according to a combination of instructions on Pharmacia leaflet on

isoelectric focusing and LKB application note 250. Leakage of the gel was prevented by sealing the edges of glass plate with agar.

3.2.11.3 Measurement of Generated pH Gradient

Immediately after completion of electrofocusing, the pH was measured with a surface pH electrode at 1 cm intervals at 10°C.

3.2.11.4 Detection of Albumins

The gel was electrofocused for a further 15 min. after measurement of the pH gradient to restore sharpness of band. The proteins were then fixed in sulphosalicyclic acid (5% w/v) and TCA (10% w/v) for 1 h. Carrier ampholytes were removed in destaining solution methanol/acetic acid/ distilled water (3:1:6 v/v) for 0.5 h and then stained in Coomassie Blue R-250[14].

3.2.11.5 Analytical Electrofocusing in Gel Rods

Electrofocusing of defatted[5] double albumins in gel rods (0.25 x 8 cm) was carried out at constant current (1 mA/gel rod) for about 3 h at room temperature. The polyacrylamide gel (T = 5%, C = 3%) contained Pharmalyte pH interval 4-6.5. The pH gradient was determined by elution of carrier ampholytes from focused gel rod (blank) which did not contain any added protein.

3.2.11.6 Measurement of pH Gradient

The blank gel rod was sliced at 1.5 mm intervals immediately after completion of electrofocusing. Each slice was then sligtly homogenised in degassed distilled water (1 ml) and ampholytes eluted with mild mechanical agitation. After about 6 h, the pH of the solution was measured with a micro pH electrode.

Table 3.2 Comparison of the electrophoretic mobilities of Albumins Mundey, Kashmir, Hussain, Birmingham[3] and Vancouver[8] on six standard media

Media	Variant Albumin	Electrophoretic mobility* of albumins		Albumin (%)		Concentration (g/l)	
		Normal	Slow	Normal	Slow	Protein	Albumin
Cellulose Acetate (cellagram)	Mundey	100	90	50	50	76	46
	Kashmir	100	90	48	52	92	46
	Hussain	100	89	46	54	80	45
	Birmingham	100	92	-	-	-	-
	Vancouver	100	89.5	-	-	-	-
Helena Cellulose Acetate	Mundey	100	93	47	53	76	43
	Kashmir†	100	91.5	49	51	92	43
	Hussain	100	92	46	54	80	47
	Birmingham	-	-	-	-	-	-
	Vancouver	100	89.5	-	-	-	-
Paper	Mundey	100-87	85-74	-	-	76	-
	Kashmir	-	-	-	-	92	-
	Hussain	100-89	87-77	-	-	80	-
	Birmingham	100	91	-	-	-	-
	Vancouver	‡	‡	-	-	-	-
Agar	Mundey	100-93	92-82	49	51	76	43
	Kashmir	100-92	91-81	47	53	92	42
	Hussain	100-94	92-84	48	52	80	55
	Birmingham	100	92	-	-	-	-
	Vancouver	100	92	-	-	-	-
Agarose	Mundey	100-94	91-85	51	49	76	52
	Kashmir†	100-93.5	90.5-84	51	49	92	52
	Hussain	100-93	89-82	50	50	80	50
	Birmingham	100	94	-	-	-	-
	Vancouver	100-90.5	89.5-81	-	-	-	-
Polyacryl-amide	Mundey	100-95	92-86	-	-	76	-
	Kashmir	-	-	-	-	92	-
	Hussain	100-95	92-87	-	-	80	-
	Birmingham	100-94	85-92	-	-	-	-
	Vancouver	100-94	93-84.5	-	-	-	-

*Electrophoretic mobility expressed on a 0-100 scale
0 = edge of cathodic serum protein
100 = edge of normal albumin

†Data was an average of two experiments

‡Albumins migrated as a single band

-Not Determined

3.2.11.7 Detection of Protein

The proteins were fixed in sulphosalicyclic acid (5% w/v) and TCA (15% w/v) overnight and then stained by Fast Green (0.25% w/v) in destaining solution acetic acid/ethanol/water (1:3:6 v/v/v). Destaining was at 40°C.

3.2 RESULTS AND DISCUSSION

The results of the electrophoretic test on Table 3.2 show that Albumin Mundey is a slow albumin variant. Its mobility is similar to Albumin Kashmir only on cellulose acetate, to Albumin Hussain on agar and polyacrylamide and to Albumins Birmingham and Vancouver on agar. Data on the electrophoretic mobility of Albumin Kashmir on paper and acrylamide were not available for a complete comparison.

These small differences in electrophoretic mobilities, due to the overall ionic charge on the albumin molecules, are of significance in distinguishing these nearly similar slow allormophs of normal HSA. A comparison of their proximity to normal albumin shows that Albumin Mundey is closest on Helena cellulose acetate and agarose while Albumin Birmingham is closest on cellulose acetate and paper. On polyacrylamide, Albumin Vancouver is nearest to normal albumin. It also shows that Helena cellulose acetate and agarose are the most sensitive media to differentiate between these variants, while agar was the least sensitive, since four of the five variants have identical electrophoretic mobility on this medium.

Table 3.3 Comparisons of the ligand binding capacities of Albumins Mundey, Kashmir Hussain, Birmingham[3] and Vancouver[8] as detected on the standard media

Media	Albumin Variants	Ligands					
		Bromophenol Blue	Ponceau S	HABA	Bromocresol Green	Bilirubin	Congo Red
Cellulose Acetate (Cellagram)	Mundey	N > S	A	U	N > S	N = S	O
	Kashmir	N > S	A	U	N > S	N = S	O
	Hussain	N > S	A	U	N > S	N = S	O
	Birmingham	N > S	Only N	-	N = S	N = S	-
	Vancouver	N > S	*	*	N > S	N = S	*
Helena Cellulose Acetate	Mundey	N > S	N > S	U	N = S	S > N	O
	Kashmir	N > S	N > S	U	S > N	S > N	O
	Hussain	N = S	N > S	U	S > N	S > N	O
	Birmingham	-	-	-	-	-	-
	Vancouver	N > S	*	*	Only S	Only S	-
Paper	Mundey	N = S	B	U	N > S	U	O
	Kashmir	-	-	-	-	-	-
	Hussain	N = S	B	U	N = S	U	O
	Birmingham	N > S	-	-	N = S	N = S	-
	Vancouver	-	-	-	-	-	-
Agar	Mundey	N > S	A	A	Only N	N = S	N > S
	Kashmir	N > S	A	A	Only N	N = S	N > S
	Hussain	N > S	A	U	A	U	N > S
	Birmingham	N > S	-	-	N > S	N = S	N = S
	Vancouver	N > S	*	*	N > S	N = S	N > S
Agarose	Mundey	N > S	A	U	N > S	N = S	N > S
	Kashmir	N > S	A	U	N > S	N = S	N > S
	Hussain	N > S	A	U	N > S	N = S	N > S
	Birmingham	-	-	-	-	-	-
	Vancouver	N > S	*	*	N > S	N = S	N = S
Polyacryl-amide	Mundey	N > S	N > S	U	N > S	N = S	S > N
	Kashmir	N > S	N > S	U	N > S	N = S	S > N
	Hussain	N > S	N > S	U	U	N > S	S > N
	Birmingham	-	-	-	-	-	-
	Vancouver	N = S	*	*	Only N	N = S	S > N

A = Mobility of ligand (A)head of albumins after electrophoresis

B = Mobility of ligand (B)ehind albumins after electrophoresis

N = Normal albumin

O = Dye at (O)rigin after electrophoresis

S = Slow albumin

U = Quantity of dye bound by albumins (U)ndetectable after electrophoresis

* = Ligand not bound by albumins (data did not state positions of ligands)

- = Not determined

Table 3.2 also shows the quantity of Albumin Mundey detectable on the different media. On cellulose acetate, it is equal in concentration to normal albumin. Slight increases in quantities (53% and 51%) were observed on the Helena and agar system respectively, but a slight decrease was observed on agarose (49%). These quantitative differences are too small to be of any significance and it can be concluded that these two albumins are in a 1:1 proportion in serum. A similar result was arrived at by the elution of albumins on unstained cellulose acetate strips.

The results of the ligand binding test on Table 3.3 show that Albumin Mundey binds bromophenol blue, bromocresol green and bilirubin either equally well or with decreased binding capacity when compared to normal albumin on all six electrophoretic media. HABA and Ponceau S were generally not bound to the albumins or were undetectable by eye on all six media. Congo Red was only bound to Albumin Mundey on agar, agarose and polyacrylamide. The binding affinities of Albumin Mundey for these ligands are individually discussed below.

Bromophenol blue was bound by normal albumin in larger quantity than Albumin Mundey on all media except paper, where equivalent binding was obtained. Quantification of bromophenol blue bound by the albumins on cellulose acetate (Table 3.4) shows that Albumin Mundey bound 34% while normal albumin bound 66% of the total quantity of dye.

The binding of Ponceau S by this albumin was only detectable on Helena cellulose acetate and acrylamide where normal albumin bound more Ponceau S than Albumin Mundey.

On the remaining media, this ligand moved as a separate band ahead of the albumin S, except on paper where the Ponceau S-free albumins were of faster mobility than this ligand.

It was impossible to estimate the quantity of HABA bound by these albumins on all six media because the HABA-albumin complex is either undetectable by eye or it moved as an advanced separate band on agar.

Bromocresol green was bound in lesser quantities by Albumin Mundey on all media except on Helena and agar. On Helena cellulose acetate, both albumins bound bromocresol green equally well.

It is interesting to note that Albumin Mundey had no binding affinity for this ligand on agar, where it was bound only by normal albumin. However, quantification of eluted bromocresol green bound by albumins on cellulose acetate (Table 3.4) shows a decreased binding capacity of Albumin Mundey (42%) whereas the value for normal albumin is 58%.

The binding of bilirubin by Albumin Mundey and normal albumin were equivalent on cellulose acetate, agar, agarose and acrylamide. On paper, the binding was undetectable but Albumin Mundey bound more bilirubin on the Helena system.

Congo Red was not bound to the albumins, but remained at the origin on cellulose acetate, Helena and paper. On agar and acrylamide, the normal albumin had the greater binding capacity but this was reversed on acrylamide.

Table 3.4 Quantification of eluted ligands bound to albumins after cellulose acetate electrophoresis

Dye	% dye bound to	
	Normal albumin	Albumin Mundey
Bromophenol Blue	66	34
Ponceau S	A	A
HABA	U	U
Bromocresol Green	58	42
Bilirubin	50	50
Congo Red	0	0

A = Mobility of dye ahead of albumins
U = Quantity of due bound by albumins
0 = Dye at origin

A critique on the ligand bind test was made in Section 1.6.2.5.2.

Comparisons of the ligand binding capacities of Albumin Mundey with the other Indian variants, Kashmir, Hussain, Birmingham and Vancouver (Table 3.3), show that the ligand binding capacity of Albumin Mundey is most identical to albumin Kashmir. To support this statement, their binding capacities are identical on all five media, except on paper. It was unfortunate that this data was unavailable for a complete comparison of the ligand binding capacity.

The binding capacities of Albumin Mundey for all ligands on cellulose acetate and agarose are indistinguishable from Albumins Kashmir and Hussain.

Albumin Hussain is similar to Albumin Mundey in the

binding of Ponceau S, Congo Red, bilirubin and bromophenol blue on Helena cellulose acetate, paper, agar and agarose. The similarity to Albumin Birmingham is in the binding of bromophenol blue and bilirubin on cellulose acetate and agar. Albumin Vancouver binds bromophenol blue, bromocresol green and bilirubin identically to Albumin Mundey on cellulose acetate and agarose. Some similarities are also observed on agar in the binding of bromophenol blue and Congo Red.

These results are summarised on Table 3.5.

Table 3.5 Similarities in ligand binding capacities of Albumins Birmingham, Hussain, Kashmir and Vancouver to Albumin Mundey

Ligand	Electrophoretic Media					
	Cellulose Acetate	Helena	Paper	Agar	Agarose	Polyacryl-amide
Bromophenol Blue	B,H,K,V	K,V	H	B,H,K,V	H,K,V	H,K
Ponceau S	*	H,K	*	*	*	H,K
HABA	†	†	†	*,†	†	†
Bromocresol Green	H,K,V	K	None	K	H,K,V	K
Bilirubin	B,H,K,V	H,K	†	B,K,V	H,K,V	K
Congo Red	*	*	*	H,K,V	H,K	H,K,V

B = Albumin Birmingham

H = Albumin Hussain

K = Albumin Kashmir

V = Albumin Vancouver

* = Ligand was not bound to albumins

† = Quantity of bound ligand was undetectable

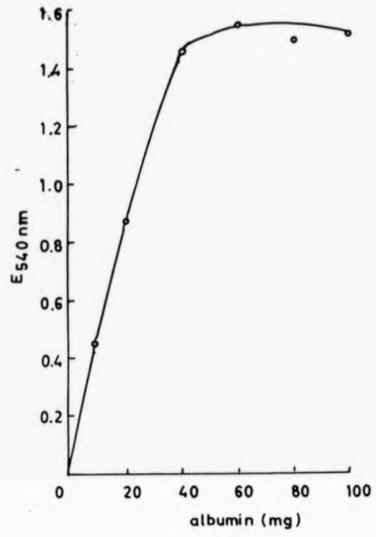

Fig. 3.1 Biuret Protein Standard Curve

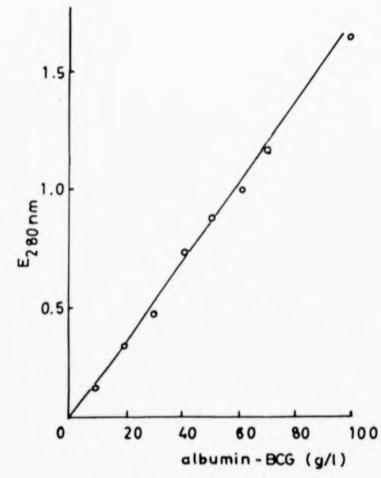

Fig. 3.2 Bromocresol Green Standard Curve

These comparisons of mobility and ligand binding suggest that Albumin Mundey may be an identical variant to Albumin Kashmir. However, a firm conclusion could not be made even though their qualitative binding capacities (Table 3.3) may be identical on all test media. A quantitative survey may reveal important differences in the amount of ligands bound by these two variants. Also, a comparison of their electrophoretic mobility (Table 3.2) does not correlate on all six media.

The total concentration of serum proteins of the healthy subject a year after hospitalisation was 92 g/l (Fig. 3.1). This was considerably higher than the value (76 g/l) measured during hospitalisation (Table 3.2).

The albumin concentration was 52 g/l as measured by improved analytical estimation of albumin using bromocresol green (Fig. 3.2). This value was also considerably increased from the average concentration of 46 g/l obtained during hospitalisation (Table 3.2). Both protein and albumin concentrations are known to decrease during illness[18] but are restored to their normal value after recovery. Since the ratio of the albumins is 1:1 (Table 3.2), the concentration of both normal and abnormal albumins is about 26 g/l respectively.

The electrophoretic mobility and concentration of Albumin Mundey was unchanged after repeated freezing, thawing and heating. This was proof of its thermal stability and that its slow electrophoretic mobility was not due to bound charged ligands that could be removed by these thermal processes, as found in some albumin variants discussed in Section 1.6.2.6.2.

SDS-PAGE showed that Albumin Mundey was of similar relative molecular mass as normal albumin. The same conclusion was arrived at by gradient PAGE and this confirmed that Albumin Mundey is a monomeric variant.

Mild limited tryptic digestion of Albumin Mundey (isolated and in serum) showed an unchanged electrophoretic mobility when compared to the control Albumin Mundey. Under identical conditions, the propeptides of the slow variants, Albumins Christchurch and Lille were cleaved to reveal albumins of similar electrophoretic mobility to normal albumin.

Albumin Mundey shares a common antigenity with normal albumin. A single immunoprecipitation line was formed by the reaction of this variant against monospecific anti-normal HSA as shown on Fig. 3.3.

Fig. 3.3 Immunoprecipitation of albumins against anti-normal HSA
Sample well: (A) normal HSA, (B) Albumin Mundey
The central well contained anti-normal human albumin antisera. (The bubbles were formed because of the heat of the photographic plate.)

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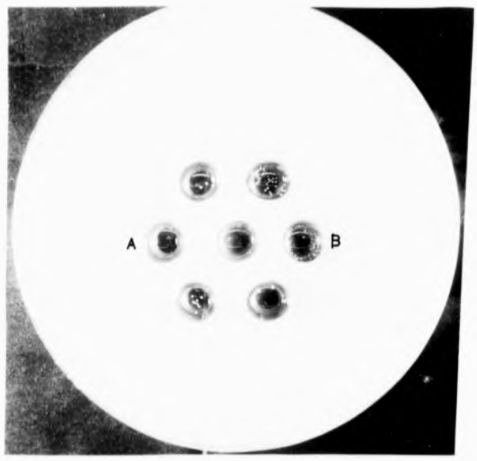

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Sample well: (A) normal HSA, (B) Albumin Mundey
The central well contained anti-normal human albumin antisera. (The bubbles were formed because of the heat of the photographic plate.)

The immunoprecipitation band of the double albumins in sera were slightly wider (figure not shown) than that of the single albumins as on Fig. 3.3. This band was twice as close to the central antisera well than the immunoprecipitation band of the control normal albumin. Although Albumin Mundey reacted with anti-normal antisera, this did not prove total immunochemical similarities to normal albumins. The reasons are discussed in Section 1.5.7. Reaction with specific monoclonal antibodies (Section 1.6.2.6.4) should be a more sensitive method to detect minute immunochemical differences.

On the electrofocusing gel shown on Fig. 3.4, Albumin Mundey is identified as the second most anionic of the serum proteins.

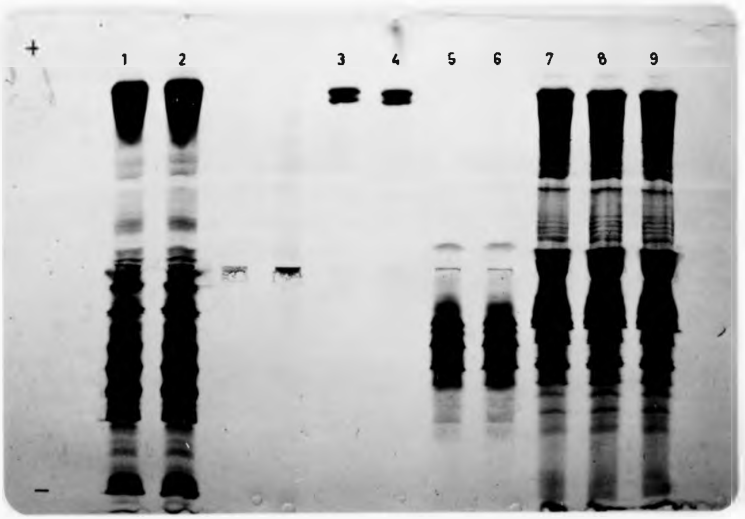

Fig. 3.4 Isoelectric focusing of various proteins in the pH 3-10 range
Samples: (1,2,7,8,9) normal serum; (3,4) chromatographically pure unseparated Albumin Mundey and normal albumin; (5,6) precipitation of Mundey's serum by ammonium sulphate (0-45%).

The immunoprecipitation band of the double albumins in sera were slightly wider (figure not shown) than that of the single albumins as on Fig. 3.3. This band was twice as close to the central antisera well than the immunoprecipitation band of the control normal albumin. Although Albumin Mundey reacted with anti-normal antisera, this did not prove total immunochemical similarities to normal albumins. The reasons are discussed in Section 1.5.7. Reaction with specific monoclonal antibodies (Section 1.6.2.6.4) should be a more sensitive method to detect minute immunochemical differences.

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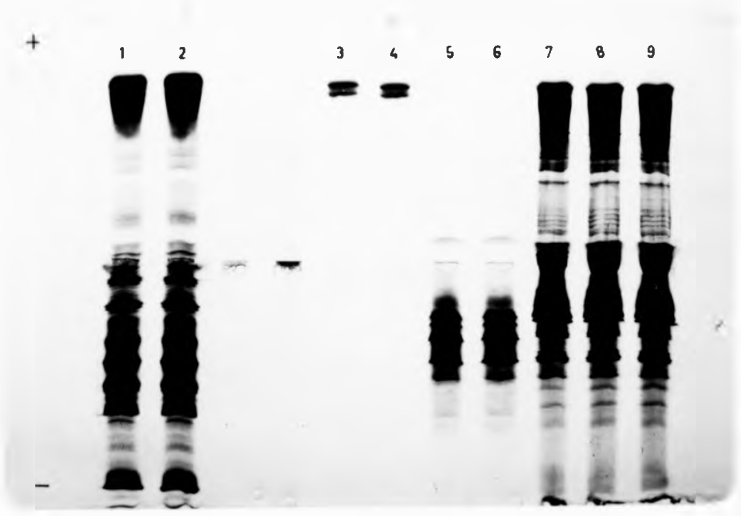

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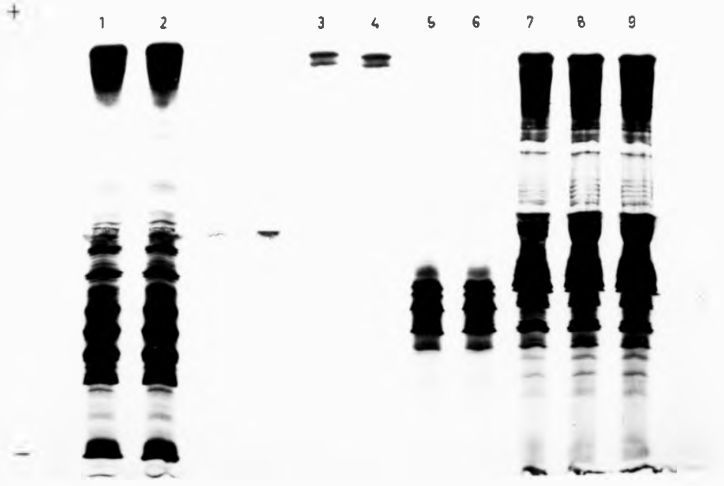

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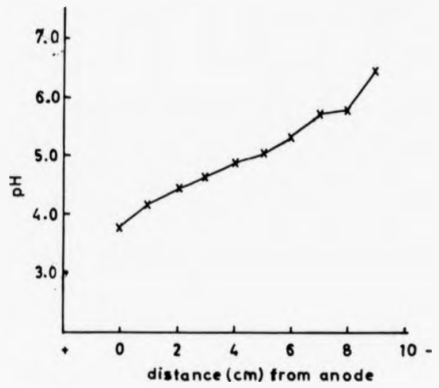

Fig. 3.5 pH profile of thin-layer isoelectric focusing gel focused in the pH 4-6.5 range.

Analytical thin-layer isoelectric focusing in the pH range 4-6.5 (Fig. 3.5) showed that the isoelectric point of the defatted and native Albumin Mundey to be 5.7 and 5.0 respectively. The charge difference between these two moieties and normal albumin (also defatted and native) was about 0.1 pH units. Isoelectric focusing in gel rods between the same range (Fig. 3.6) of defatted Albumin Mundey shows the isoelectric point to be 5.7.

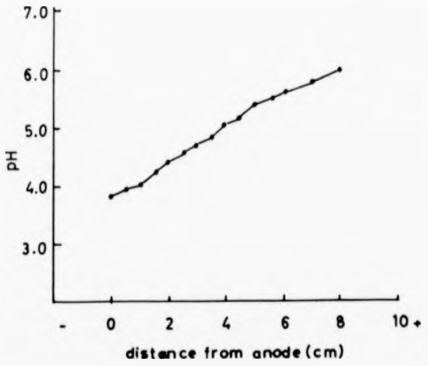

Fig. 3.6 pH profile of gel rods focused in the pH 4-6.5 range.

The result of these tests show that Albumin Mundey is a heat-stable monomeric variant present in an equal quantity to normal albumin in serum. It does not have any additional peptide at the N- or C-terminal ends of the albumin peptide chain and is genetically transmitted with Gc-2. Double immunoprecipitation with anti-normal human albumin antisera demonstrate immunochemical similarities to normal albumin. Defatted Albumin Mundey is isoelectric at pH 5.7 but the native species is isoelectric at pH 5.0. The electrophoretic mobility of Albumin Mundey was identical to several Indian variants on different media. The dye binding test shows the greatest similarity in ligand binding capacity to Albumin Kashmir.

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CHAPTER IV

STAGE 2: SEPARATION OF ALBUMIN MUNDEY FROM NORMAL ALBUMIN

The physiochemical properties of Albumin Mundey are almost identical to those of normal albumin. This lack of distinction was the source of great difficulties in effecting their separation, indeed, it was once thought to be impossible. This chapter summarises the techniques tried (with disappointing results) over the span of one year.

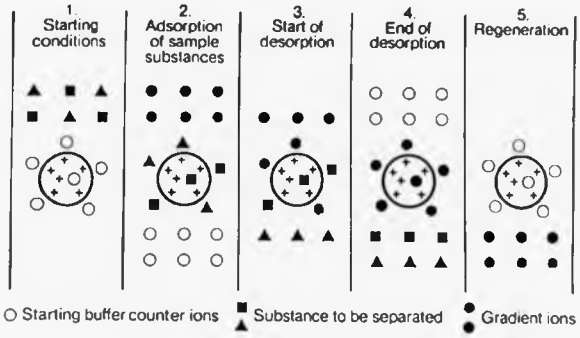
The principles of the methods used in attempting separation, could only exploit the very subtle differences between the albumins. These differences are: (i) ionic charge (ion-exchange chromatography and polyacrylamide gel electrophoresis); (ii) isoelectric points (isoelectric focusing and chromatofocusing); and finally (iii) affinity chromatography based on their differential specific binding affinities for dyes of different chemical structures.

The methods used in this difficult and highly laborious separation are described below.

4.1 ION-EXCHANGE CHROMATOGRAPHY

Marginally different protein allomorphs, differing in as little as a single amino acid, are supposedly separable by this technique (Section 1.6.2.6.5).

However, an inherent problem in this technique



Stage 1 shows the ion exchanger in equilibrium with its counter-ions. Sample substances are about to enter the ion exchange bed. In stage 2 the counter-ions have been exchanged for sample substances. After this adsorption a gradient is applied. Desorption of one sample species occurs at stage 3. This substance is exchanged for counter-ions in the eluting buffer and is therefore eluted from the ion exchanger. At stage 4 the remaining sample substance is exchanged for gradient ions and eluted, after which regeneration may be started. The gradient ions are exchanged for counter-ions in stage 5 and the ion exchanger is thus regenerated and ready for re-use.

Fig. 4.1 Principles of ion-exchange chromatography

is that the amino acids of albumin (isolated and in serum) can easily undergo acid-to-base-transformations. There are many ways in which this can arise, including storage (private communication of Dr. S. G. Franklin to Dr. D. W. Hutchinson). The result is to further decrease, or even nullify, the already small ionic charge difference between the albumins. This can hinder their separation by this technique.

Another problem is the oxidation of sulphur in thioether linkage to the corresponding sulphoxide. This results in many different chromatographic forms of albumin with varying quantities of sulphoxide groups. These forms deter the chromatographic separation of the albumins which should only comprise two pure forms; normal and abnormal. Thiodiglycol, an antioxidant, is

usually incorporated into chromatographic buffers (0.1 to 0.3% v/v) to keep the thioether sulphur in the reduced state. Other amino acid transformations that can occur in peptides are discussed in Section 6.1.

The variable parameters in ion-exchange chromatography used to attempt separation include chromatography at pH 5.2[2], 5.6, 5.7, 5.75, 6.8, 7.0, 7.5 and 8.8. These pH values were chosen to be intermediate and at increasing pH units above their isoelectric points of about 5.7 (Section 3.2). The last pH value of 8.8 was tried since separation by electrophoretic techniques was achieved at this pH.

These systems were coupled with a continuous, linear decreasing salt gradient. In the decreasing pH system[1], the pH range was chosen such that the isoelectric points of the albumins coincided with the approximate centre of the pH gradient. These separations were generally performed on long thin chromatographic columns with only small quantities of albumins applied, to aid their separation. The continuous elution of the albumins from one such column to two other parallel columns, of similar dimensions, was also tried.

4.2 AFFINITY CHROMATOGRAPHY

Albumin Mundey differs from normal albumin in its specific binding affinities of certain ligands (Table 3.3). This property may be sufficiently discriminating so as to effect the rapid and quantitative separation of Albumin Mundey by affinity chromatography (Fig. 4.2).

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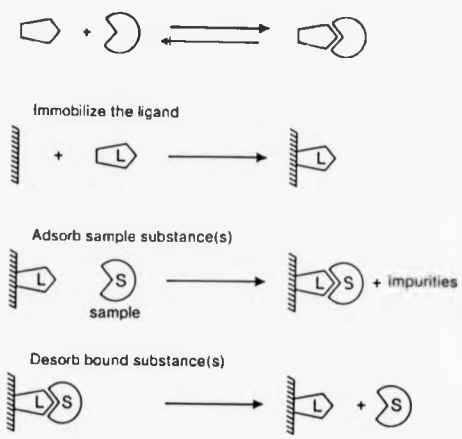
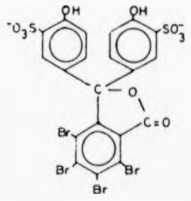


Fig. 4.2 Principle of affinity chromatography

Albumin Mundey was screened against 65 affinity columns in the laboratory of Dr. P. G. Dean (University of Liverpool). Each affinity column consisted of a specific dye covalently attached to Sepharose CL6B. The amphipathic dye bromosulphophthalein (19) was immobilised onto Sepharose CL4B and the chromatographic behaviour of normal albumin then tested on this column.



(19)

4.3 ISOELECTRIC FOCUSING

In this technique, the separation of the albumin relied solely on the slight difference in their isoelectric

points. Preparative isoelectric focusing in the pH 4-6.5 range was tried in a granulated gel medium as well as in polyacrylamide. Fig. 4.3 illustrates the principles of the technique.

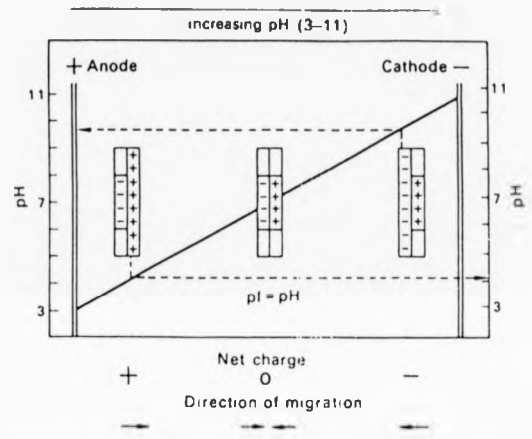


Fig. 4.3 Schematic drawing illustrating the principles of isoelectric focusing

4.4 CHROMATOFOCUSING

Chromatofocusing is isoelectric focusing on ion-exchanges without the application of an external potential[13]. In this technique, the chromatographic gel is initially equilibrated with the buffer of the higher pH value in the pH gradient. A gradient is then generated by the gradual titration of this *in situ* buffer with a second amphoteric buffer. The pH profile of the chromatographic column shows a gradual linear decrease in pH down the length.

The journey of a protein down this column is a continual cycle of attachment, followed by detachment from

the gel and migration down the column, depending on the charge of the protein at that instant (Fig. 4.4).

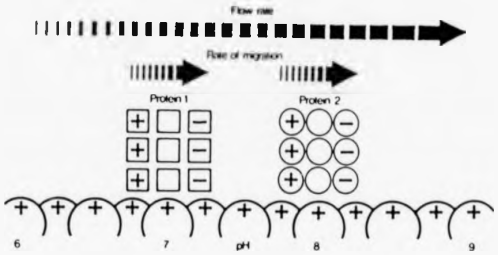


Fig. 4.4 Behaviour of a protein in chromatofocusing

This charge is determined by a combination of its isoelectric point (pI) and the pH of the surrounding buffer. When the pH of the buffer is above the pI, the protein becomes negatively-charged and so binds to the cationic exchanger. When the changing pH drops below the pI of the protein, it becomes positively charged and is repelled by the exchanger. It then travels further down the column until the combination of buffer and pI causes it once again to be attached to the gel.

An attempt to separate the two albumins, with a difference in isoelectric points of only 0.1 pH units by chromatofocusing was attempted in the pH 6-4 range. Recycling of the relevant peak to obtain better distinction was also tried.

4.5 PREPARATIVE PAGE

This was the only successful method in which quantitative isolation of Albumin Mundey was achieved.

It is a rapid method (can be completed in a day) and does not require expensive chemicals or equipment. A bonus is that high yields of pure albumins can be obtained (over 90%).

The double albumins, obtained by ammonium sulphate precipitation[8] and ion-exchange chromatography, were first separated on an anodic disc-PAGE system[4,14].

After electrophoresis they were rapidly stained with the fluorescent dye, 1-anilinonaphthalene-8-sulphonic acid[7]. This stain was chosen because albumins bound this dye instantly and the usual lengthy staining and destaining processes normally used in PAGE were avoided. Since protein bands become less distinct in the absence of an electric field, the use of this stain afforded the immediate visualisation of the albumins after electrophoresis. Therefore it ensured that the two close albumin bands could be excised before they merged as one blurred, indistinct band.

To extract the albumins from the gel slice, reverse polarity electrophoresis was employed. The method of Otto and Snejdatkova[10] was used with gel rods and on vertical slab gels, an adaptation of this method by Mendel-Hartig[9] was used. The eluted albumin contained tightly bound dye molecules (Section 1.5.5.2) which were removed by interaction with charcoal at low pH[3].

4.6 ANALYSIS OF THE ALBUMIN FRACTIONS

The albumin fractions produced by these preparative methods were analysed by cellulose acetate electrophoresis, disc-PAGE[4,14] and ultrathin isoelectric focusing between

pH 4-6.5[5]. The latter two methods were excellent in detecting the extent of separation of the albumins. Cellulose acetate electrophoresis at pH 8.6 was initially used to detect separation of albumins immediately after a separation process, but the wide blurred albumin bands were observed on electrophoresis. This effect is due to the salts in the sample and electrolyte interacting with the albumins.

4.7 EXPERIMENTAL

4.7.1 By ion-exchange chromatography

The general method used involved the application of small quantities of salt-free double albumins (about 10 mg) or serum (3 ml) on long thin chromatographic columns (100-150 length x 1.5 cm i.d.). The anion exchangers were DEAE-Sephadex, DEAE-Sephacel or QAE-Sephadex. The eluting buffers were sodium acetate, *tris*-HCl or sodium phosphate at I = 0.1. The pH values of these buffers were pH 5.2[2], 5.60, 5.70, 5.75, 6.8, 7.0, 7.5 and 8.8.

After running the dissolved double albumins or serum into the gel, the flow rate of the buffer was decreased to almost zero. After 30 min., the columns were washed with buffer to remove unattached proteins. The adsorbed proteins were eluted with a continuous linear NaCl gradient (0-0.5 M) in the same buffer. Individual fractions from various points in the peaks were subjected to analysis by the technique mentioned in

Section 4.6. After this, the albumins were desalted by dialysis against water and then lyophilised.

4.7.1.1 By Decreasing pH-gradient[1]

About 50 mg of double albumins were applied to a QAE-Sephadex column (24 x 4 cm i.d.) equilibrated with phosphate buffer (pH 5.9, 0.1 M). The adsorped albumins were then eluted by the application of a decreasing pH gradient (pH 5.9 to 5.5).

4.7.1.2 By Continuous Elution into Parallel Columns

Double albumins (50 mg) were loaded onto a QAE-Sephadex column (106 x 1.5 cm i.d.) equilibrated with *tris*-HCl (pH 6.5, 0.2 M). The outlet of this column was connected by tubing to a second column of identical composition and dimensions. Connection to a third column was made in a similar fashion. The albumins were desorbed by application of a linear NaCl gradient (0-0.5 M).

4.7.2 Preparative electrofocusing in granulated gel

LKB application note 198.

4.7.2.1 Preparation of Electrofocusing Gel bed

The granulated gel bed was prepared by adding Ultrodex (4.0 g) slowly to Pharmalyte pH range 4-6.5 (5.3 ml) in water (95 ml) to form a 4% (w/v) gel slurry. After pouring into the electrofocusing tray, the calculated quantity of water was evaporated from the slurry by means of a small fan placed about 80 cm directly above the tray. When the correct consistency was reached, the tray was

transferred to the cooling plate of LKB Multiphor 2117. Good thermal contact between the electrofocusing tray and the cooling plate was ensured by the application of a thin film of Triton X-100 (0.1% w/v). The pH gradient was established at 8 W for 0.75 h with H_3PO_4 (1 M) as anode buffer and NaOH (1 M) as cathode buffer.

4.7.2.2 Electrofocusing

The salt-free double albumins (120 mg) were dissolved in 3 ml of Pharmalyte (pH 4-6.5) solution (0.5 ml in 10 ml water). The sample well was prepared by pressing a sample applicator at the approximate centre of the gel and removing its contents. A sample slurry was formed by mixing the removed sample gel with the solution of double albumins. This mixture was returned to the sample well. After hydrostatic equilibration, electrofocusing was carried out at 10 W for 16 h at 10°C. After this, the gel was fractionated by a grid of 0.8 cm slit widths.

4.7.2.3 Detection and Elution of Protein

After electrofocusing, the albumins were detected by the contact print method of Radola[12]. The pH profile of the gel was obtained by measuring at 0.8 cm intervals with a surface electrode. The gel from each grid was transferred into micro-chromatographic columns and the albumins eluted from each gel with equal volumes of water. The absorbance of the eluate of each column at 280 nm was measured. The albumin-containing peaks were applied on Sephadex G-50 columns to remove the ampholyte carriers. They were then concentrated *in vacuo* before analysis by ultra-thin isoelectric focusing.

4.7.3 By preparative isoelectric focusing in PAGE

The double albumins were subjected to electrofocusing in PAGE rods (0.6 x 7 cm) containing Pharmalyte pH 4-6.5/water (1:15 v/v) in polyacrylamide gel (T = 5%, C = 3%). The anodic buffer was DL-glutamic acid (0.01 M) and cathodic buffer L-histidine (0.01 M).

Prefocusing of the gel to establish the pH gradient was at 1 mA/gel. Electrofocusing was at 1 mA/gel using myoglobin (pI = 5.6-8.0) as marker protein. After electrofocusing, the albumins in a gel were located by rapid staining in Fast green (0.25% w/v) in acetic acid/ethanol/water (1:3:6 v/v/v) for 1 h, without prior fixation of albumins. The destained gel was then aligned with the unstained gels and the corresponding albumin bands excised using a clean degreased razor. The gel slices containing abnormal albumin were homogenised in water. The homogenate was magnetically stirred at $4^{\circ}C$ for 12 h. The polyacrylamide was then precipitated by centrifugation on a bench top centrifuge. Small traces of polyacrylamide in the supernatant were removed by passage through a medium-pore sinter under suction. The carrier ampholyte from the resulting solid-free supernatant was removed by gel filtration on Sephadex G-75 followed by lyophilisation.

4.7.4 By chromatofocusing

All buffers and gels used in this technique were thoroughly degassed to prevent fluctuations in

the pH gradient. This effect, due to dissolved atmospheric CO_2, occurs between pH 5.5 to 6.5. The anionic exchanger, PBE 94, in a water-jacketed column (26.5 x 1.5 cm) was equilibrated with the starting buffer, L-histidine-hydrochloride (pH 6.2, 0.025 M). The column packing was checked by the migration of cytochrome C (4 mg, horse-heart type IIA).

The pH of the column was adjusted with the amphoteric eluting buffer, Polybuffer-hydrochloride (5 ml, 0.0094 mmol/pH unit/ml) prior to sample application to prevent exposure of the sample to vast pH differences. The mixture of the albumins (120 mg), which was dialysed against the starting buffer for 48 h at 4°C, was applied to the column. Elution was at a constant flow rate (30 ml/h) using a peristatic pump. A pH gradient (pH 6.2-4.0) was generated by the application of twelve column volumes (360 ml) of the eluent. Fractions (3 ml) were monitored at 280 nm and the pH of every fifth fraction recorded.

After concentration of the relevant albumin peaks, Polybuffer was removed from the individual albumin peak by gel filtration on Sephadex G-75 eluted with the start buffer. After concentration *in vacuo* at 30°C, the first albumin peak which should contain the slow alloalbumin was subjected to rechromatography under identical conditions to remove normal albumin contained in the sample.

Chromatofocusing of the double albumins (60 mg) in the narrow pH range (5-4) was also tried. The water-jacketed column (40 x 1 cm i.d.) was equilibrated with

degassed piperazine-HCl (pH 5.5, 0.025 M). The pH gradient was generated by titration with Polybuffer 74 (pH 4.0, 0.075 mmol/pH unit/ml Polybuffer) at a flow rate of 32.2 ml/h.

4.7.5 By affinity chromatography

4.7.5.1 Dye-Affinity Chromatography[15]

The dye-affinity column was synthesised as follows.

Sepharose CL6B (50 ml) was suspended in an equal volumn of water warmed to 60°C. The dye (50 mg in 5 ml water) was added with vigorous stirring followed after 15 min. by addition of NaCl (5 g). The mixture was then heated to 80°C and solid Na_2CO_3 (1 g) was added, followed by incubation at the same temperature for 30 min. The dyed gel was then washed with hot water (80°C).

The affinity of the double albumins for the dyes was tested as follows.

The dye-affinity gel (1 ml) was washed with 6 M urea/0.5 M NaOH (15 ml) and then equilibrated with the eluting buffer, sodium phosphate (20 ml, pH 8.0, 20 mM). The purity of the colum was checked by measuring the absorbances of the eluate at 280 nm. The double albumins (1 mg) were applied and the column washed with ten column volumes of buffer. The absorbance of the eluted albumins at 280 nm was measured.

The albumins were then desorbed by five column volumes of sodium phosphate (pH 8.0, 20 mM) containing

NaSCN (0.2 M) and KCl (1 M). The absorbance of the fractions at 280 nm was measured. This step was repeated to desorb any remaining albumin. The control affinity column was Sepharose CL6B without any attached dyes.

4.7.5.2 By Bromosulphophthalein Affinity Chromatography[6]

Sepharose CL4B (20 ml) was washed copiously with water and then equilibrated in NaOH (4 ml, 5 M) for 10 min. Bromosulphophthalein (3.04 g) and $NaBH_4$ (160 mg) were added and the suspension refluxed with gentle stirring for 5 h at 80°C. The attached gel was then washed consecutively with NaOH (1 M), glycine (0.1 M)/KCl (1 M) (pH 3), SDS (2%), urea (6 M), KSCN (2 M) in sodium phosphate (pH 7.4, 0.01 M), distilled water and finally phosphate buffer (pH 7.4, 0.01 M) containing EDTA (0.001 M) and NaCl (0.1 M).

Normal albumin was applied to this affinity column equilibrated with buffer [KCl (0.1 M), phosphate (0.01 M) and EDTA (0.001 M) pH 7.40]. After eluting the column with this buffer to remove unabsorbed albumins, a linear gradient of 0-1.2 M KSCN was used to elute the albumins.

4.7.6 By preparative PAGE under non-denaturating conditions

4.7.6.1 Separation of Albumin

The double albumins obtained by ammonium sulphate precipitation[8] and purification by ion-exchange chromatography were separated by disc-PAGE[4,14] system.

The separating gel was *tris*-(0.375 M)-HCl (0.06 M) (pH 8.9) in polyacrylamide (T = 7.5%, C = 2.6%). The stacking gel was *tris*-(0.062 M)-HCl (0.062M) (pH 8.3) in polyacrylamide (T = 4.5%. C = 2.5%). The electrolyte was *tris*-(0.05 M)-glycine (0.38 M) (pH 8.3). The separation can be achieved in gel rods (0.9 x 8 cm) or vertical slabs (17 x 16 x 1 cm).

Separation of mixed albumins (5 mg/gel rod) in ultrapure sucrose (10%, 200 µl) was achieved by electrophoresis at 2 mA/gel for 30 min. and then 3 mA/gel for 1.5 h.

4.7.6.2 Staining Procedure[7]

Immediately after electrophoresis, the gels were stained in 1-anilino-8-naphthalene (0.003%, Mg^{2+} salt) in sodium phosphate buffer (pH 6.8, 0.1 M) for about 1-2 min. The albumin bands were excised with a clean degreased razor under long wavelength u.v. (365 nm). Although it was usually unnecessary, the fluorescence could be enhanced by exposing the gel to conc. HCl fumes or immersion in HCl (3 M) for a few seconds.

4.7.6.3 Electrophoretic Elution of Albumins[9,10]

The slice of albumin containing gel was placed on top of a polyacrylamide support gel. This gel of similar composition to the separating gel was initially subjected to pre-electrophoresis at 2 mA/gel rod for 3 h. A buffer [400 µl, 0.025 M *tris*/0.075 M glycine and 30% glycerol] was layered above the tube. The other void areas above the gel and buffer were filled with NaCl (2 M). Elution of the protein from the gel was at reversed polarity at 6 mA/gel rod for 3 h. The complete elution of albumin from the gel was checked by observing the gel slice for

any remaining fluorescence under UV light. After electrophoresis, the NaCl layers were carefully syringed off and the albumin-containing fractions dialysed against several changes of deionised water at 4°C. This fraction, after concentrating, when necessary, was subjected to the charcoal treatment of Chen[3] to remove the tightly bound fluorescent dye and fatty acids. On slab gels, the gel slice was 'glued' to the support gel by the addition of unpolymerised acrylamide. After polymerisation, the electrophoretic elution of the albumin was carried out as described above.

4.7.7 Analysis of the albumin fractions

4.7.7.1 Ultrathin Isoelectric focusing[5]

An ultrathin (0.36 mm) isoelectric focusing polyacrylamide gel (T = 5%, C = 3%) in pH range 4-6.5 was polymerised into a chamber consisting of a U-shaped gasket made of three films of Parafilm between a silanised glass plate and an untreated glass plate. The outer edges of the glass plates were sealed with agar to prevent leakage. During pouring of the deaerated polymerising solution, two paper clips were wedged in between the upper edges of the glass plate. These clips were removed during polymerisation. Isoelectric focusing, on LKB Multiphor, was performed in the conventional way: the anode solution was DL-glutamic acid (0.04 M); the cathode L-histidine (0.2 M). 5 µl of the albumin solution [albumin (0.5% w/v) dissolved in sucrose (10%)] was applied into the sample

wells on the gel. Electrofocusing was at 400 V for 30 min., 1000 V for 30 min. and 1500 V for 15 min. using a LKB 2197 power supply.

After focusing, the proteins were fixed in 50% sulphosalicyclic acid/10% TCA for 30 min. The Pharmalyte carriers were removed in destaining solutions, methanol/acetic acid/water (3:1:6 v/v/v) for 30 min. Coomassie Brilliant Blue R-250 (0.2%) in destaining solution was used to stain the gel. Destaining was in the destaining solution. A permanent record of the gel was obtained by pressing a filter paper gently on the surface of the gel. After a few minutes, the paper was gradually pulled back with the gel attached to the paper. It was then dried on a makeshift frame.

4.7.7.2 Disc-PAGE

The method of Tárnoky and Dowding[14] was used.

4.7.7.3 Cellulose Acetate Electrophoresis

Electrophoresis on cellulose acetate was carried out in barbital buffer (pH 8.6, 0.05 M) at 12-25 V/cm for 50 min. (width-wise) or 120 min. (length-wise). Staining of the cellulose acetate strip was in Ponceau S (0.2% w/v) in TCA (3%) for 10 min. followed by three successive washes in acetic acid 5% (v/v).

4.8 RESULTS AND DISCUSSION

Ion exchange chromatography should resolve the double albumins into two peaks if separation into the normal and abnormal albumin had been achieved. These

two peaks need not be completely separate. However, the chromatography of the double albumins on DEAE- and QAE-Sephadex columns at pH 5.2, 5.60, 5.70, 5.75, 6.8, 7.0 and 7.5 at different gel bed heights failed to separate the albumins.

The UV absorbance profile of the albumin fractions at 280 nm showed only a single peak, instead of the expected two. Analysis of fractions at either side of this single peak by thin layer isoelectric focusing at pH 4-6.5 showed double albumin bands. Since all the precautions (i.e. packing, sample preparation and size, elution, etc) to ensure good chromatographic separation were undertaken, one may only conclude that the albumins are so similar as to escape separation under these conditions.

The only instance where insignificant separation was achieved was on a DEAE-Sephacel column equilibrated with *tris*-HCl buffer (pH 8.8, 0.1 M). The elution profile is shown on Fig. 4.5.

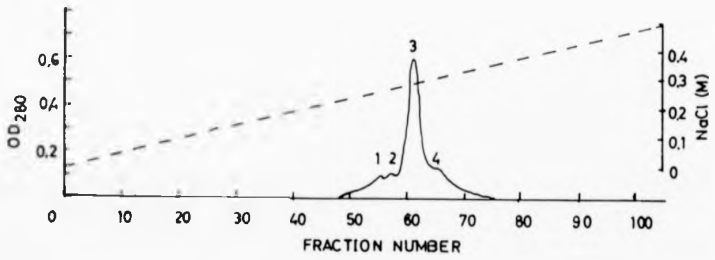


Fig. 4.5 Elution pattern of double albumins on DEAE-Sephacel
Column: 100 x 3 cm, Eluent: *tris*-HCl (0.1 M, pH 8.8) with linear NaCl gradient to 0.5 M. Each fraction: 10 ml.

The minor peaks 1 and 2 consisted of pure Albumin Mundey while the mixture of albumins was detected in the major peak 3. Peak 4 also consisted of double albumins but contained a slightly lesser quantity of the abnormal moiety. Therefore, ion-exchange chromatography was generally an ineffective method in the preparative isolation of Albumin Mundey.

Fig. 4.6 shows the positions of the albumins after electrofocusing in a granulated gel medium. The albumins were focused as two prominent peaks (A and B) of approximate isoelectric points (pI_e), 5.0 and 5.6. Peak A actually consists of two minor peaks (i and ii) of approximate pI_e of 4.8 and 5.0.

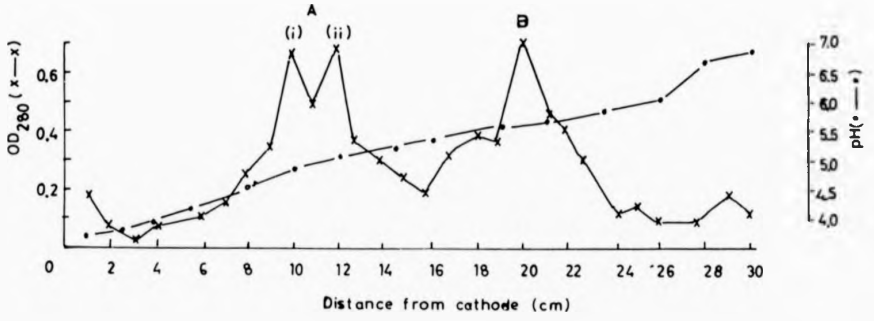


Fig. 4.6 Preparative isoelectric focusing of the double albumins in the pH range 4-6.5

In Section 3.2, the defatted double albumins were determined to be isoelectric at pH 5.70 and 5.71. Therefore they were expected to be focused around the region of peak B. Analysis of various fractions of peaks A and B by ultrathin isoelectric focusing between pH 4 and 6.5 showed that separation was not effected.

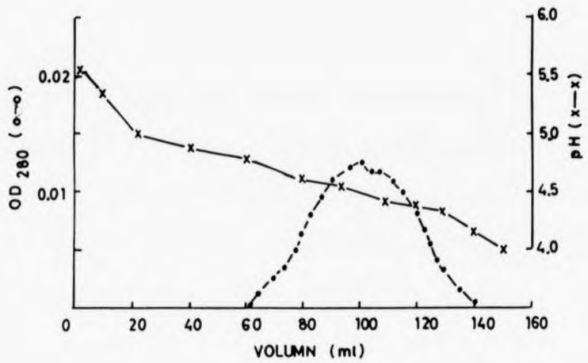


Fig. 4.7 Chromatofocusing of double albumins between pH 5 to 4.

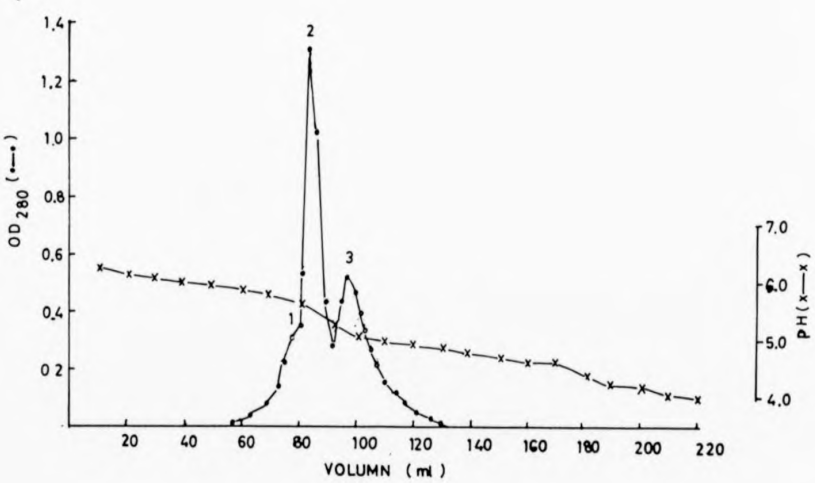


Fig. 4.8 Chromatofocusing of double albumins between pH 6 to 4

It was not possible to explain why the albumins focused at two vastly different pI's. The possibility that incomplete electrofocusing may be a factor can be discounted, since a pH gradient (about 3.5 to 6.8) was established as shown on Fig. 4.6. The measured pH at the extremities of the gel bed was slightly below and above the focusing range of pH 4 to 6.5 due to the effect of the electrolytes located at these positions.

Although Albumin Mundey was obtained by preparative isoelectric focusing on polyacrylamide, the percentage recovery of albumins obtained by free diffusion of the albumins from homogenised gels was very low. Only about 10% of the proteins were recovered. Another source of loss was the removal of Pharmalyte carriers by gel filtration.

If the double albumins were separated by chromatofocusing, Albumin Mundey should be eluted before normal albumin since the pI_e of Albumin Mundey is the higher of the two albumins. Therefore, it is fractionated as the leading band in a decreasing pH gradient.

Chromatofocusing of the double albumins in the narrow pH gradient (5 to 4) and the wide pH gradient (6 to 4) are shown on Figs. 4.7 and 4.8, respectively. Better resolution was obtained in the wide pH range.

The elution profile of the albumins between pH 6 to 4 (Fig. 4.8) shows two main peaks numbered 2 and 3. Peak 1 is a poorly resolved peak at the shoulder of peak 2. When fractions of these peaks were analysed by thin layer isoelectric focusing, Albumin Mundey was eluted first, as expected (peak 1). Peak 2 consisted of mixed albumins

as did peak 3. The latter consisted of a slightly lesser quantity of Albumin Mundey.

It appeared that rechromatography of peak 2, consisting of double albumins, under identical conditions to the first should fractionate more pure Albumin Mundey. Recycling of peak 2 confirmed this expectation. Although repetitive recycling of the second peak from each successive chromatofocusing should collectively produce pure Albumin Mundey, heavy losses in material can be expected due to successive rechromatofocusing and also to the intermediate process of removing the Polybuffer by gel filtration. As this study was actually performed simultaneously with preparative PAGE, this latter method proved to be faster, inexpensive and more productive.

The chromatographic behaviour of Albumin Mundey on 65 dye-affinity columns was monitored for two factors. The first factor was the binding affinity of the double albumins for the immobilised dye. The second was the differential binding of the albumins, that is, the quantity of albumin, (as measured by absorbance at 280 nm) eluted before and during block elution with buffers containing sodium thiocyanate (0.2 M) and potassium chloride (1 M) to desorb the albumins. Dyes that were considered to be useful in the separation of the albumins would desorb half of the total quantity of albumin before the block elution and the remaining half during this process. Dyes that manifested this behaviour are listed on Table 4.1. The analysis of these two fractions obtained from each of the dyes listed on this table showed the unseparated double albumins.

Table 4.1 Immobilised dyes that selectively bound the double albumins

Procion Red	MX-5B, H-8BN, H-3B, HE-3B, MX-G, H-3BN
Procion Blue	H-B-3R, H-5R, MX-G, MX-2G, HE-RD, MX-4GD
Procion Yellow	H-5G, HE-3G, MX-GR, H-3R
Procion Green	HE-4BD
Procion Brown	H-2G, MX-3BR, H-5BR
Procion Rubine	MX-B
Procion Scarlet	MX-G
Procion Turquoise	H-7G
Procion Olive	H-7G
Procion Orange	MX-2R
Procion Navy	MX-RB, H-4R
Cibacron Olive	G-P
Cibacron Brilliant Green	4G-A
Cibacron Brilliant Yellow	3G-P
Cibacron Brilliant Blue	BRP
Cibacron Black	BGA

Bromosulphthalein binds serum albumin with high affinity in free solution[11]. However, when this dye was bound onto an insoluble matrix, it did not bind normal albumin which was used as a test sample. The reason may be that steric hindrance prevented binding.

The last point in this Chapter is the report of the successful separation and isolation of Albumin Mundey in high quantitative yields. This isolation of

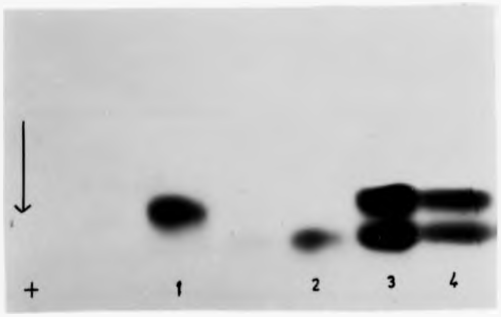


Fig. 4.9 Analytical disc-PAGE[14] on albumins obtained by preparative PAGE[4,14]
Sample (1) Albumin Mundey, (2) normal albumin, (3,4) unseparated Albumin Mundey and normal albumin. Sample loading (1) 20 µg, (2) 10 µg, (3) 50 µg, (4) 30 µg.

Albumin Mundey in a pure, homogenous form as shown on Fig. 4.9, facilitated progression of this study to the third stage, namely fragmentation studies to detect the mutant peptide.

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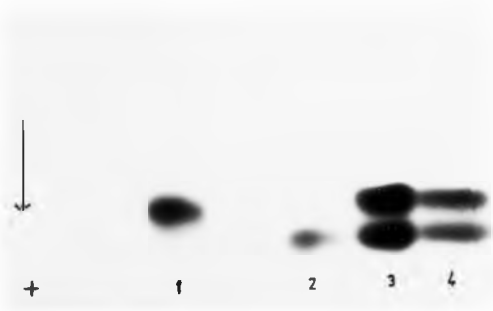

Fig. 4.9 Analytical disc-PAGE[14] on albumins obtained by preparative PAGE[4,14]
Sample (1) Albumin Mundey, (2) normal albumin, (3,4) unseparated Albumin Mundey and normal albumin. Sample loading (1) 20 µg, (2) 10 µg, (3) 50 µg, (4) 30 µg.

Albumin Mundey in a pure, homogenous form as shown on Fig. 4.9, facilitated progression of this study to the third stage, namely fragmentation studies to detect the mutant peptide.

4.9 REFERENCES

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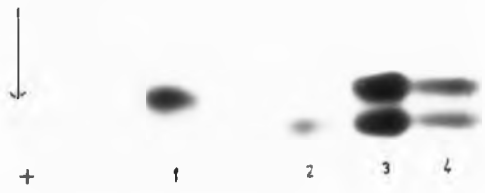

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CHAPTER V

STAGE 3: FRAGMENTATION STUDIES ON ALBUMIN MUNDEY

The last chapter described the preparative isolation of Albumin Mundey in a highly purified form. This chapter describes methods used in end group determinations and chemical fragmentations of Albumin Mundey at the methionines and tryptophan residues.

The N-terminal residue was determined by labelling with DABITC[4] and dansyl chloride[11]. The C-terminal residue was identified by reaction with carboxypeptidase Y[17].

The methionine-specific reagent CNBr* and the tryptophan-specific reagents, NBS†, NCS‡ and *o*-iodosobenzoic acid, were used to fragment the albumin into relatively large peptides.

The products of these fragmentations were carefully monitored over numerous experiments, under standardised experimental conditions, for consistency in the fragmentation patterns (number of peptides produced). Cleavages at tryptophan were abandoned in the later stages of sequencing because of the lack of specificity. Instead CNBr was used because of its unequalled high specificity, quantitative efficiency and reproducibility. Amino acid modifications of proteins purified by chromatography are known to occur

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*CNBr = Cyanogen bromide
†NBS = N-bromosuccinimide
‡NCS = N-chlorosuccinimide

as described in Section 6.1. With this possibility in mind, both Albumin Mundey, processed by chromatography and PAGE, and the albumins in serum were subjected to cleavage. Their fragmentation patternson PAGE were analysed for differences.

The control, normal HSA, was isolated from serum in an ultrapure form by a triple chromatographic process. Before subjection to fragmentations all the albumins were defatted[5], reduced and S-carboxymethylated[6], except for reaction with *o*-iodosobenzoic acid where S-pyridylethylation[15] was used.

5.1 REDUCTION AND S-CARBOXYMETHYLATION OF ALBUMIN[6]

The seventeen disulphide bridges of albumin were reduced with dithiothreitol in the presence of a denaturant (8 M urea) and EDTA. The sulphydryl groups formed were alkylated by *o*-iodoacetic acid to the anionic S-carboxymethyl cysteine residue (Scheme 5.1). The reaction of this halogen with the thiol group is a

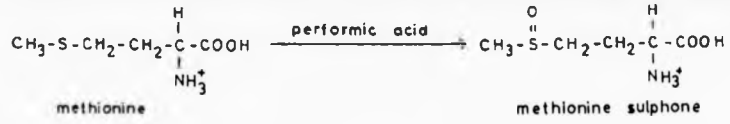
$$\text{Protein-SH} + \text{I-CH}_2\text{COOH} \longrightarrow \text{Protein-S-CH}_2\text{COOH} + \text{HI}.$$

Scheme 5.1

bimolecular nucleophilic substitution reaction where the highly reactive mercaptide ion is the nucleophilic reagent[2], EDTA was required to chelate heavy metal ions so that the catalytic reoxidation of the sulphydryl group was prevented.

5.2 PERFORMIC ACID OXIDATION

Initially performic acid oxidation of cystines and cysteines to cysteic acid was used[27]. However, this process destroyed tryptophan and methionine residues which were essential for the chemical cleavage of albumin. Performic acid rendered albumin resistant to degradation by CNBr by oxidising methionine to the stable sulphone as shown in Scheme 5.2. Tryptophan was converted to N'-formylkynurenine and other degradation products.



Scheme 5.2

5.3 N-TERMINAL RESIDUE DETERMINATION OF ALBUMIN MUNDEY

The general principle of N-terminal residue determination is based on the introduction of a marker group (fluorescent, coloured or UV absorbing) onto the amino function followed by characterisation of the derivatised amino acid. This N-terminal residue could have been determined by several methods. These are reactions with dinitrofluorobenzene[26], dansyl chloride[14], 4-N,N-dimethylaminoazobenzene-4'-isothiocyanate*4, or by the cyanate method[31].

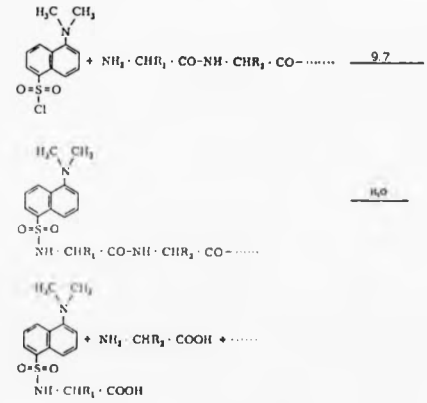
---

*4-N,N-dimethylaminoazobenzene-4'-isothiocyanate = DABITC

The N-terminal residue of Albumin Mundey was eventually determined by the dansyl chloride[11] and DABITC[4] methods. These methods were chosen because of their simplicity in methodology and high sensitivity at the nanomole and picomole level respectively. This determination was also used to detect the presence of any non-albumin proteins, but not of normal albumin since these residues may be identical.

5.3.1 Labelling with dansyl chloride

The non-fluorescent dansyl chloride (1-dimethyl-aminonaphthalene-5-sulphonyl chloride) gives a strong yellow fluorescence when it forms a sulphonamide with the amino groups of amino acids and N-terminal residues. This is described on Scheme 5.3. The optimum molar ratio of N-terminal



Scheme 5.3

residue to dansyl chloride for efficient labelling is 1:5 at pH 9.7 and 37°C. Dansyl chloride is hydrolysed to

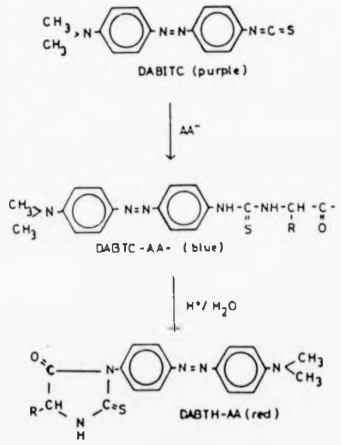
DNS-OH* by water and by hydroxyl ions. It is easily identified as a large blue spot. In the presence of excess reagent, DNS-NH_2** is probably produced as shown in Scheme 5.4.

$$\text{DNS-NH} \cdot \text{CHR} \cdot \text{COO}^{\ominus} + \text{DNS-Cl} \rightarrow \text{DNS-NH} \cdot \text{CHR} \cdot \text{COO-DNS} \xrightarrow{\text{OH}^{\ominus}} \text{DNS-NH}_2 + \text{RCHO} + \text{CO}_2 + \text{DNS-OH}$$

Scheme 5.4

5.3.2 Labelling with DABITC

DABITC couples with the amino-group of the N-terminal residue in alkaline solution to form the DABTC***-peptide (Scheme 5.5). This moiety then cyclises to form the red DABTH****-amino acid in aqueous solution. This amino acid derivative is easily detectable on chromatograms as a coloured moiety.



Scheme 5.5

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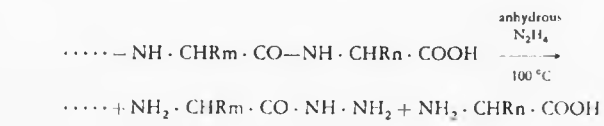
*DNS-OH = 1-dimethylaminonaphthalene-5-sulphonic acid
**DNS-NH_2 = 1-dimethylaminonaphthalene-5-sulphonamide
***DABTC = 4-N,N-dimethylaminoazobenzene-4'-thiocarbamoyl
****DABTH = 4-N,N-dimethylaminoazobenzene-4'-thiohydantoin

5.4 C-TERMINAL RESIDUE DETERMINATION

The choice of chemical methods for C-terminal analysis is more limited than for N-terminal analysis. Instead the endopeptidases, carboxypeptidase A, B, C and Y, are a popular choice.

Some chemical methods involve selectively labelling the C-terminal residue with tritium[20] or by hydrazinolysis[1]. The latter method was initially chosen but was later abandoned because of explosive side-reactions. Anhydrous hydrazine is used in rocket propulsion fluid! During hydrazinolysis, amino acid hydrazides are released from the C-terminal of the protein, but only the C-terminal residue is liberated as the free amino acid. These moieties are released when the protein is heated with anhydrous hydrazine at 100°C.



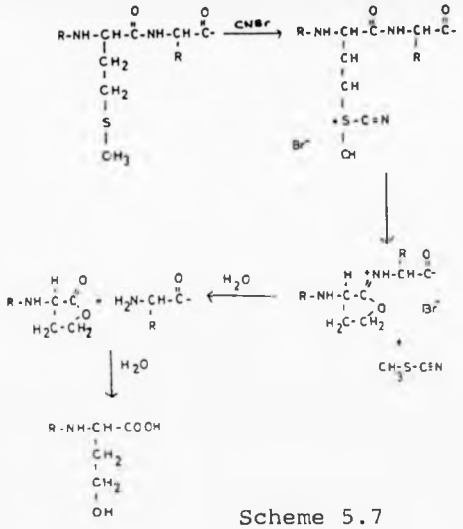
$$\cdots\cdots\text{NH}\cdot\text{CHRm}\cdot\text{CO}\text{—NH}\cdot\text{CHRn}\cdot\text{COOH} \longrightarrow \cdots\cdots + \text{NH}_2\cdot\text{CHRm}\cdot\text{CO}\cdot\text{NH}\cdot\text{NH}_2 + \text{NH}_2\cdot\text{CHRn}\cdot\text{COOH}$$

Scheme 5.6

Carboxypeptidase Y (EC 3.4.12.-) an exopeptidase of the 'serine' and 'acid' class, was used to determine the C-terminal residue of Albumin Mundey. This enzyme has a broad specificity and releases amino acids sequentially from the C-terminus of proteins and peptides. The C-terminal residue was then identified by TLC.

5.5 SPECIFIC CLEAVAGE AT METHIONINE BY CNBr[12]

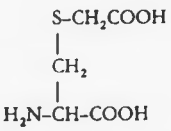
CNBr cleaves the C-terminal bond of methionine by reacting specifically with the sulphur on the thioester side chain of methionine. A mixture of homoserine, homoserine lactone and methylthiocyanate is formed. Therefore, the C-terminal residues of all fragments produced will be homoserine or its lactone except of course, when methionine is C-terminal (Scheme 5.7).



Scheme 5.7

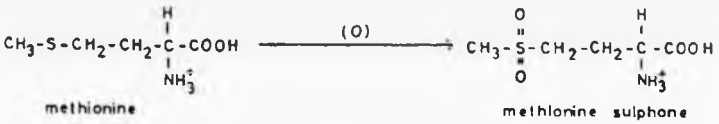
Side reactions that can occur in acidic conditions include:

(i) The attack of cysteinyl side chains without bond cleavage. Carboxymethylation of the cysteinyl side chain to S-carboxymethyl-cysteine (20) prevented this.



(20)

(ii) Methionyl side chains can be oxidised to the sulphoxide or sulphone (Scheme 5.8). But this effect was checked by maintaining the reaction mixture in an oxygen-free atmosphere.



Scheme 5.8

CNBr cleavage of HSA at the six methionines should produce seven peptides if all the bonds were equally susceptible to cleavage. However, methionine-123 is only partially susceptible to cleavage. When cleavage at this residue does not occur, methionine is converted to homoserine[7]. When this factor was taken into account in this cleavage, eight peptides were expected as cleavage products (Table 5.1).

5.6 CLEAVAGE AT TRYPTOPHAN

HSA only has one tryptophan residue at position 214. Therefore, specific cleavage at this residue should, ideally, produce two fragments of quite distinct sizes and character. It was originally hoped that if the appro-

TABLE 5.1 Fragmentation Products of HSA by CNBr

CNBr Fragment Number	N-terminal Residues	Residue Number*
I	Aspartic acid	1-87
II†	Alanine	88-123
II + III†	Alanine	88-298
III	Cysteine	123-298
IV	Proline	299-329
V	Phenylalanine	330-446
VI	Proline	447-548
VII	Aspartic acid	549-585

*Primary sequence of HSA according to Meloun[23]
†Fragments produced by partial cleavage at Met-123

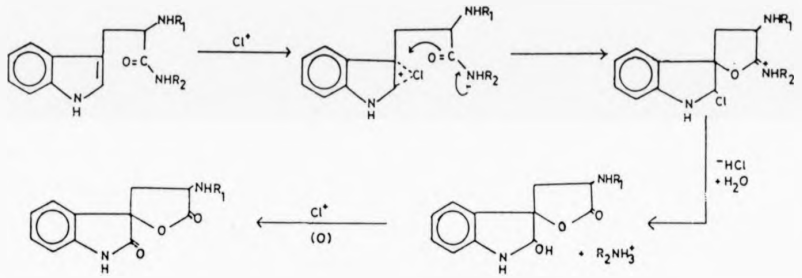
priate half of Albumin Mundey could be identified by electrophoresis to contain the mutation(s), it could then be isolated and then specifically degraded again into smaller fragments (e.g. by trypsin and/or CNBr). By this second cleavage, smaller mutant fragment(s) could then be identified. The identification of the small fragment(s) could be done by realignment of the peptides by overlapping sequences followed by determination of the N-terminal residues.

The reagents used (NBS, NCS and *o*-iodosobenzoic acid) were found generally to lack specificity, gave low cleavage yields and were accompanied by undesirable side reaction, unless performed under very stringent conditions. Albumin Mundey unseparated from normal albumin was subjected to tryptophan-cleaving reagents since these experiments

were performed before their very difficult separation had been achieved.

5.6.1 With NBS and NCS

The mechanisms of cleavage by NBS and NCS (Scheme 5.9) are similar at low pH [24,27], whereby they halogenate



Scheme 5.9

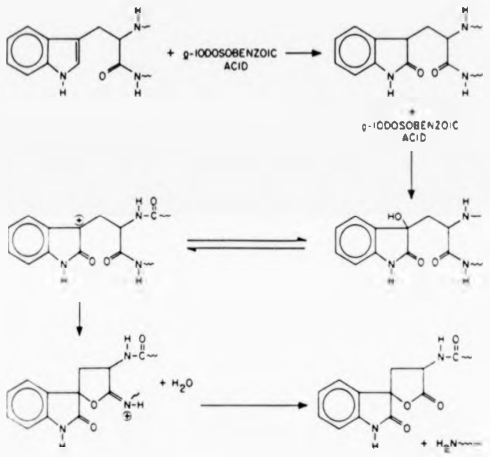
the indole nucleus. The halogenated indole moiety then undergoes spontaneous dehalogenation to form the oxindole derivatives through a series of oxidation and hydrolysis. The oxindole derivative then promotes the cleavage reaction.

Side reactions that can occur with NBS cleavage are (i) oxidations of sulphydryl groups and disulphide bonds, which can result in cleavage of cysteinyl bonds[30]; and (ii) the increased susceptibility of tyrosyl and histidyl bondsto cleavage in larger excess of NBS[25].

5.6.2 With *o*-iodosobenzoic acid

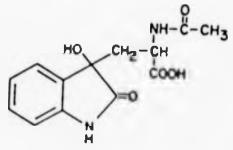
The proposed mechanism of cleavage by *o*-iodosobenzoic acid is generally similar to that of NBS and NCS[25] except that a halogenated intermediate is not formed.

The mechanism on Scheme 5.10 consists of four



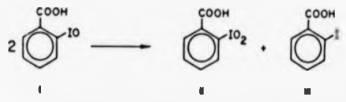
Scheme 5.10

separate steps. The first oxidation step produces the oxindolyl derivative[28]. The following oxidation step produces an iminospirolactone formed by cyclisation of the carbonyl carbon on the oxidised indole nucleus. This step is probably the rate-determining step. The last step is the hydrolysis of the iminolactone to form N-acyldioxindolyalanine (21) resulting in peptide bond cleavage.



(21)

Oxidation of tyrosyl residues by *o*-iodoxybenzoic acid, which is the disproportionate product of *o*-iodosobenzoic acid (Scheme 5.11), can presumably be reduced by preincubation with *p*-cresol. *p*-Cresol reacts with the released I^+ or I_2[19].



Disproportionation of two molecules of *o*-iodosobenzoic acid (I) produces one molecule of *o*-iodoxybenzoic acid (II) and one molecule of *o*-iodobenzoic acid (III).

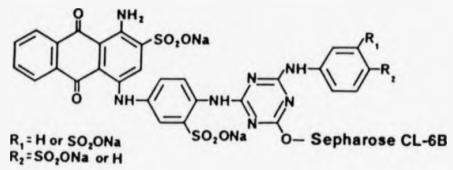
Scheme 5.11

5.7 Preparation of chromatographically pure normal albumin

Normal HSA was used as a control in all these experiments. The successive methods used to obtain chromatographically pure normal HSA from pooled serum were: ammonium sulphate precipitation[22], ion exchange chromatography, affinity chromatography and gel filtration.

Precipitation of albumins from serum by ammonium sulphate rather than dextran sulphate[21] or polyethylene glycol[10] was chosen because of the great ease of removal of the precipitating agent[16]. Contaminants such as γ-globulins and bilirubin-bound albumin were removed by ion-exchange chromatography on QAE-Sephadex. Affinity chromatography on Sepharose CL6B (22) removed the last traces of non-albumin proteins. Albumin dimers and oligomers were removed by gel filtration on Sephadex G-100. The resulting yield of ultrapure albumin was 30%. This albumin was then defatted by the method of

Chen[5]. The purity of the albumin was determined by SDS-PAGE[34] and conventional thin layer PAGE.



(22)

5.8 EXPERIMENTAL

5.8.1 Preparation of chromatographically pure normal HSA

5.8.1.1 Ammonium Sulphate Precipitation

Method modified from McMenamy[22]. Ammonium sulphate (2.77 g) was added slowly with mixing to serum (10 ml) contained in a centrifuge tube to form a 45% saturated solution. The pH was adjusted to 6.5. After standing at 4°C for 5 min., the proteins were precipitated by centrifugation on a bench top centrifuge. The supernatant was removed into another centrifuge tube to which ammonium sulphate (2.10 g) was slowly added to form a 75% solution. The albumins were pelleted by centrifugation and then semi-dried by inversion of the tube on a piece of filter paper. The albumin was solubilised in water (about 10 ml) and the pH adjusted to 6.5. It was then dialysed against *tris*-HCl (pH 7.0, 0.01 M) at 4°C by ultrafiltration at a pressure of 15 psi in a magnetically-stirred cell fitted with an Amicon PM-10

filter. The albumin was then subjected to ion exchange chromatography.

5.8.1.2 Ion-Exchange Chromatography

The ammonium sulphate-precipitated albumin was subjected to continuous gradient elution ion-exchange chromatography on a QAE-Sephadex column. The column (40 x 2 cm i.d.) was equilibrated with *tris*-HCl (pH 7.0, 0.1 M). Initially the flow rate of the column was adjusted to near zero for half an hour. It was then washed with five column volumes of loading buffer until the absorbance of the eluate at 280 nm was near zero. Desorption of the albumin was by a continuous linear gradient of NaCl (0-0.5 M). The albumin peaks were then dialysed against *tris*-HCL (pH 7.0, 0.05 M) and then applied to affinity chromatography.

5.8.1.3 Affinity Chromatography

The albumins were applied to a water-jacketed Blue Sepharose CL6B affinity column (10 x 1.5 cm i.d.) equilibrated with *tris*-HCl (pH 7.0, 0.05 M) at a very slow flow rate. After zero flow rate for about half an hour, the unabsorped proteins were eluted off. The chromatographically pure albumin was desorbed by block elution with the eluting buffer containing KCl (1.5 M).

5.8.1.4 Gel Filtration

Gel filtration of the albumin was on a Sephadex G-100 (100 x 1.5 cm i.d.) column eluted with water. The albumin peaks were dialysed against water (1.5 l) and then lyophilised.

5.8.1.5 Defatting and Removal of Bound Ligands on Albumin

The method of Chen[5] was followed exactly as published.

5.9 DETERMINATION OF PURITY OF ALBUMINS

5.9.1 By SDS-PAGE

The purity of albumin was determined by slight overloading of the proteins on SDS-PAGE[34].

5.9.2 By conventional thin-layer PAGE (LKB application note 306)

Large numbers of albumin samples were screened by horizontal thin layer PAGE (26 x 11.5 cm). This consisted of a *tris*-glycine buffer (75.1 g glycine and 2.5 g sodium azide dissolved in 3 l water, then titrated to pH 8.9 with *trisma* base) in polyacrylamide (T = 7.7%, C = 2.6%). Pre-electrophoresis was at 50 mA for 30 min. at 10°C on LKB Multiphor 2117. Albumin samples (15 mg/ml) were applied onto the gel *via* filter paper sample holders. Electrophoresis was at 15 V/cm for 2.5 h. The albumins were fixed in TCA (57.0 g)/sulphosalicyclic acid (17.0 g) in methanol/water (500 ml, 3:7 v/v) for 1 h at room temperature. The gel was stained in Coomassie Blue R-250 (0.1%) in methanol/acetic acid/ water (5:1:5 v/v/v) and then destained in ethanol/acetic acid/water (3:10:6 v/v/v).

5.10 END GROUP DETERMINATION

5.10.1 Reduction and S-carboxymethylation[6]

Deionised, cyanide ion free urea (3.60 g) and EDTA (15 mg) in *tris*-HCl (12 ml, pH 8.4, 1 M) were rendered oxygen-free by bubbling in oxygen-free nitrogen (having passed through alkaline pyrogallol) for about 30 min. in a screw-cap vial. Pure normal or abnormal albumin (25 mg) was added. Water (2 ml) and urea [4.5 ml, 8 M in 0.2% (w/v) EDTA] were added to fill the tube completely. β-mercaptoethanol (100 µl) was added for reduction at room temperature for 4 h.

The albumins were S-carboxymethylated by dropwise addition of iodoacetamide (0.268 g) in NaOH (1 ml, 1 M). The sample was then dialysed against deionised water containing thiodiglycol (0.2%) and EDTA (0.02%).

5.10.2 Dansyl chloride method[11]

The reduced and S-carboxymethylated albumin (50 µg), was dissolved in SDS (1%, 50 µl) in a test tube (0.4 x 5 cm) by immersion in a boiling water bath for 5 min. N-ethylmorphorline (50 µl) was added to the cooled mixture followed by dansyl chloride (75 µl, 25 mg/ml) in anhydrous dimethylformamide to label the N-terminal residue. Dansylation was achieved by letting the reaction mixture stand at room temperature for 3 h, after which dry acetone (500 µl) was added to precipitate the protein.

After about 30 min., the mixture was centrifuged and the pellet obtained redissolved and washed in dry acetone (80%, 500 µl), and then dried *in vacuo*.

The product was hydrolysed by heating in 5.7 M hydrochloric acid at 105°C for 18 h. in the flame - sealed tube. After drying *in vacuo* at 40°C, dry pyridine was added (50%, 10 µl) to the sample. The labelled N-terminal residue was determined by two-dimensional ascending TLC on micropolyamide plates. The solvent systems used [1.5% formic acid and benzene/acetic acid (4.5:1 v/v)] were by Lee and Safille[18] or Hartley[13]. Fluorescence of the dansylated derivatives under UV light (365 nm) could be enhanced by exposure to concentrated ammonia fumes. The controls were normal HSA with and without added dansyl chloride. Dansyl derivatives with close proximity to dansyl-L-aspartic acid (dansyl-L-arginine, -ε-lysine, -serine and -glutamic acid) were used as markers on the chromatogram.

5.10.3 By DABITC method[4]

Albumin (1 nmol) was dissolved in 50% aqueous pyridine (80 µl) in a small test tube (0.4 x 5 cm). A freshly prepared solution of DABITC (40 µl, 4 mg/ml in pyridine) was then added. The ensuæing coupling reaction was performed in a flame-sealed glass tube at 52°C for 2.5 h, after which the excess reagent and by-products were extracted with 3 portions of heptane/ethyl acetate (500 µl, 2:1 v/v) by vortexing and centrifugation. The organic layer was carefully removed and the remaining aqueous layer dried

in a vacuum over P_2O_5. The dried sample was redissolved in water (40 µl) and acetic acid-saturated hydrochloric acid (80 µl). Cleavage of this labelled N-terminal residue was at 52°C for 50 min.

The DABITC-derivatives were identified by two dimensional ascending TLC on micro-polyamide plates. Solvent systems were water/acetic acid (2:1 v/v) and toluene/*n*-hexane/acetic acid (2:1:1 v/v/v). To develop the red colour of the DABTH-amino acid, the dried chromatograms were exposed to HCl vapour. A control, normal HSA, was also used.

5.10.4 Synthesis of control DABITC-derivatives[3]

L-amino acids (0.5 µmol) were dissolved in buffer [100 µl, acetone (25 ml), triethylamine (1.2 ml) and water (20 ml) titrated to pH 10.1 with acetic acid. The volume was made up to 50 ml with water] and incubation lasted 1 h at 30°C. The solution was then dried *in vacuo* over P_2O_5. The dried compound was then redissolved in water (200 µl) and hydrochloric acid-saturated acetic acid (400 µl). Incubation was for a further 50 min. at 50°C. After drying *in vacuo* again, ethanol (400 µl) was added to extract residue. Synthesised derivatives were L-glycine, L-arginine, L-aspartic acid, L-asparagine, and L-glutamine.

5.10.5 C-terminal determination by cleavage with carboxypeptidase Y[7]

The full enzymatic activity of the lyophilised carboxypeptidase Y (5 µg) (specific activity: 55 µmol/

min/mg against acetyl-tyrosine-ethyl ester) was initially restored by incubation with water (1 µl) at 0°C overnight. The activity of the contaminant, yeast proteinase A, in the preparation was inhibited by preincubation with a solution (10 µl) of pepstatin A (1 mM) in MES buffer (10 mM, pH 6.8) for 2 min. at room temperature.

The reduced and S-carboxymethylated albumin (50 µg) was dissolved in MES buffer (20 µl, 10 mM, pH 6.8). This sample was then added to the activated carboxypeptidase. Incubation was at 37°C for 10 min. The reaction was stopped by freezing and the mixture lyophilised. The controls used were: normal albumin with and without added carboxypeptidase Y, L-leucine and L-glycine. These two amino acids are the last and penultimate amino acid residues of normal HSA. The C-terminal residue was identified by ascending TLC on silica gel G plates (without fluorescence). The solvent system was chloroform/methanol/30% ammonia (2:2:1 v/v/v). Spots were visualised by spraying with ninhydrin (0.5%) in acetic acid/1-butanol (1:20 v/v) and then incubated at 110°C for 10 min.

5.11 SPECIFIC CLEAVAGE AT METHIONINE

5.11.1 Cleavage of Albumin Mundey

The normal albumin was dissolved in 70% formic acid to a concentration of 3 mg/ml. CNBr (50-fold molar excess) was added and the reaction allowed to proceed for 20 h at room temperature in a tightly stoppered flask.

Water (10 ml) was added to the reaction mixture and the resulting solution lyophilised to remove volatile reactants. This procedure was repeated. Thiodiglycol (2%) was added to all solutions used after cleavage. The control was Albumin Mundey without any added CNBr.

5.11.2 Cleavage of double albumins in serum

This cleavage was performed according to the method of Franklin[9]. A corresponding control serum containing no CNBr was also set up.

Fragmentation analysis was on the polyacrylamide (12%) slab gel containing urea (5 M)/Triton X-100 (6 mM)/ acetic acid (5%)[35].

5.12 CLEAVAGE OF DOUBLE ALBUMINS AT TRYPTOPHAN

5.12.1 Cleavage by NBS[8]

The double albumins (0.5 mg) were dissolved in 8 M urea/2 M acetic acid (90 µl). A twenty fold molar excess of NBS (26 mg) in the same buffer (10 µl) was added to the albumins, followed by incubation at room temperature for 20 min. The reaction was terminated with a ten molar excess of tryptophan (10 mg). The cleavage products were then separated on a Sephadex G-10 column (14 x 0.5 cm i.d.) eluted with 8 M urea. The eluate was monitored at 280 nm. The control consisted of double albumins without any NBS.

5.12.2 Cleavage by NCS[29]

The double albumins (2 µmol) were dissolved in 50% acetic acid (3 ml) and NCS (10 fold molar excess) in dimethylformamide (0.3 mmol/ml) added. The reaction mixture was stirred for 40 min. at room temperature after which methionine was added to destroy excess CNBr. The solvents were then removed *in vacuo* and the precipitate redissolved in acetic acid (0.1 M, 800 µl). The fragments were desalted on a column of Biogel P-Z (90 x 1.5 cm i.d.) eluted with acetic acid (0.1 M). The control double albumin contained all the reagents except NCS.

5.12.3 Cleavage by *o*-iodosobenzoic acid[15]

The reduced double albumins were first reacted with 4-vinyl-pyridine and then subjected to cleavage by *o*-iodosobenzoic acid.

Double albumins (0.9 µmol) dissolved in guanidine-hydrochloride (6 M)/*tris* (0.13 M)-EDTA (0.1 mg/ml) (pH 7.6, 3 ml) were reduced with dithioerythritol (52 µmol) for 3 h at room temperature. After reduction, the albumins were reacted with 4-vinyl-pyridine (156 µmol) for 90 min. The reaction solution was then acidified to pH 2.0 with 88% formic acid and then desalted by exhaustive dialysis in 88% formic acid[7].

The specificity of *o*-iodosobenzoic acid for tryptophanyl bonds was improved by decreasing the activity of *o*-iodoxybenzoic, a contaminant which modifies tyrosyl groups.

This was achieved by preincubating *o*-iodosobenzoic acid (37 µmol) with *p*-cresol in 80% acetic acid/guanidine hydrochloride (4 M) for 2.5 h at room temperature.

Double albumins (0.075 µmol) were added and the reaction mixture allowed to proceed at room temperature for 24 h in the dark. Dithioerythritol (5 mg) was added to stop the reaction.

Exhaustive dialysis in 10% acetic acid in the dark at 4°C was followed by lyophilisation. Two controls were also set up - double albumins and normal HSA, to which no *o*-iodosobenzoic acid was added.

5.13 RESULTS AND DISCUSSION

Monomeric normal HSA was eluted in peaks 2 and 4 (Fig. 5.1). The first peak represents immunoglobin G and dimeric albumins were found in peak 3, which is the shoulder of peak 2.

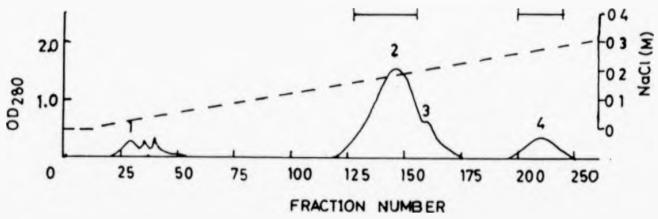


Fig. 5.1 Continuous gradient elution of ammonium sulphate - precipitated normal albumins on QAE-Sephadex A-25. Bed dimensions: 40 x 1.5 cm. Eluent: *Tris*-HCl (pH 7.0, 0.1 M). Each fraction: 2 ml.

Fig. 5.2 shows the elution profile of normal albumin on Blue Sepharose CL6B. The slight shoulder

at the start of the albumin peak may be due to bilirubin-bound albumin that escaped fractionation on the previous chromatographic column.

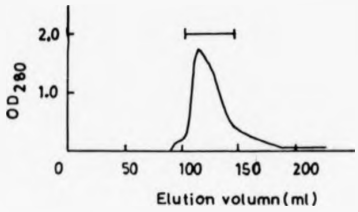


Fig. 5.2 Chromatography of purified normal albumin on Blue Sepharose CL6B. Bed dimensions: (10 x 1.5 cm). Temperature 10°C. Eluent: *tris*-HCl (pH 7.0, 0.05 M). Albumins desorped by block elution with eluent containing KCl (1.5 M).

After gel filtration, to remove aggregates of albumin, and defatting the albumins were judged to be pure by SDS-PAGE and conventional thin-layer PAGE. Fig. 3.4 illustrates its purity on an isoelectric focusing gel (pH 4-6.5).

The N-terminal residue of Albumin Mundey is L-aspartic acid. The two dimensional chromatogram (Fig. 5.3) shows an overlap of this residue with that of normal albumin and dansyl-L-aspartic acid. They are distinctly separated from other dansyl derivatives with close proximity to dansyl-L-aspartic acid. DNS-OH was recognised as a large blue fluorescent spot.

Labelling of the N-terminal residue of Albumin Mundey with DABITC produced inconclusive identification. Actually, the red DABTH-aspartic acid of normal HSA is near that of Albumin Mundey (Fig. 5.4(f)), but not to

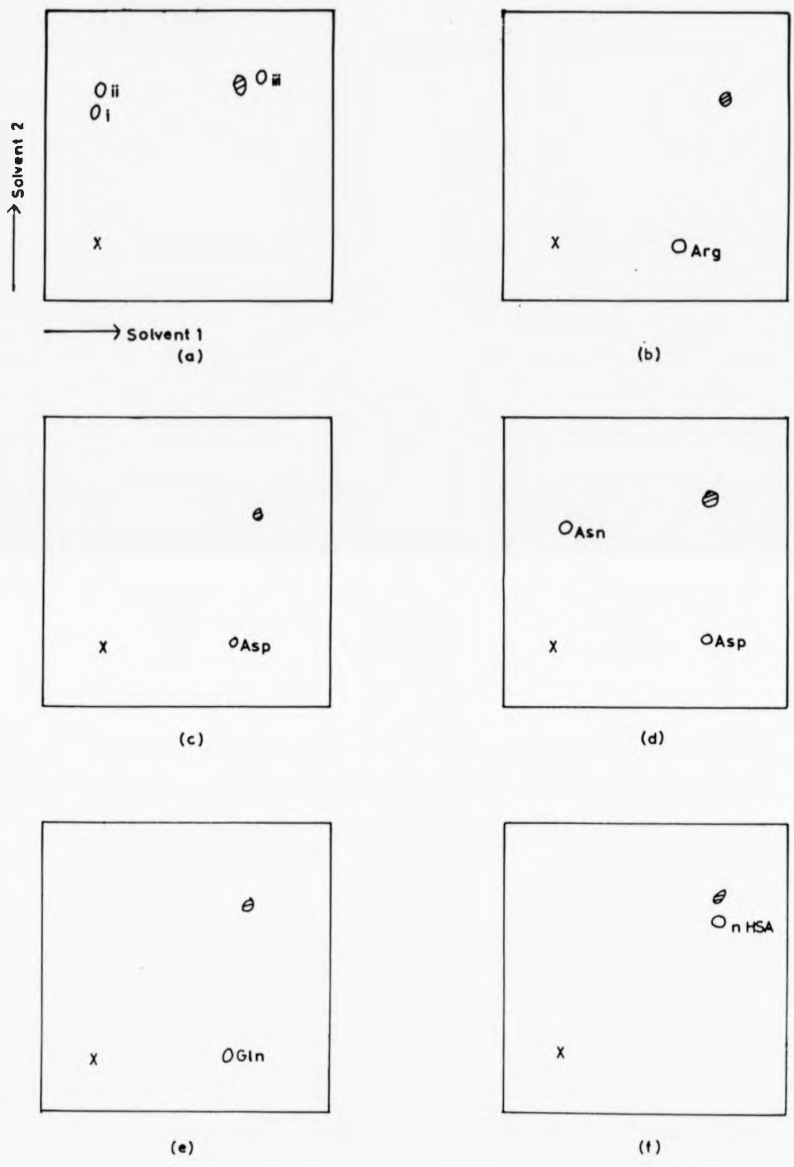


Fig. 5.4 Two dimensional chromatograms of DABTH-derivatives

(a) Lysine (b) Arginine (c) Aspartic acid
(d) Asparginine (e) Glutamine (f) Normal HSA
⊖ = N-terminal DABTH-derivative of Albumin Mundey

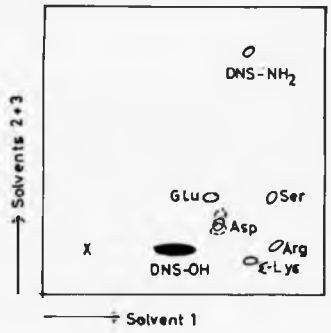


Fig. 5.3 Two dimensional chromatogram of the dansylated N-terminal residues of Albumin Mundey, the control normal HSA and other reference L-amino acids.
◌ = Albumin Mundey ◌ = normal HSA

that of the synthesised control, DABTH-aspartic acid (Fig. 5.4(c)). This latter observation may be attributed to the effect of different solvent systems used in the synthesis of the DABTH-amino acids and the labelling procedure. On Fig. 5.4(a) lysine was identified as three different moieties.

On Fig. 5.4 the red α-DABTH-ε-lys,(a)iii, with its close proximity to the N-terminal residue of Albumin Mundey, was initially thought to be the residue of interest. However, the use of the more established procedure, dansylation, disproved this without any doubt. The other two incompletely converted lysyl derivatives were identified by their colour. These are α-DABTH-ε-DABTC-lysine,(a)i,which was purple-blue and the blue α-PTH-ε-DABTC-lys,(a)ii.

Partial deamination of the asparaginyl side chain by the synthetic process resulted in the simultaneous

production of DABTH-aspartic acid. A similar partial deamination of glutamyl side chain to glutamic acid was not observed in Fig. 5.4(e).

The C-terminal residue of Albumin Mundey released by carboxypeptidase Y is L-leucine (Fig. 5.5(c). Its mobility is identical to the C-terminal residue of normal HSA (Fig. 5.5(c)) and L-leucine (Fig. 5.5(d)).

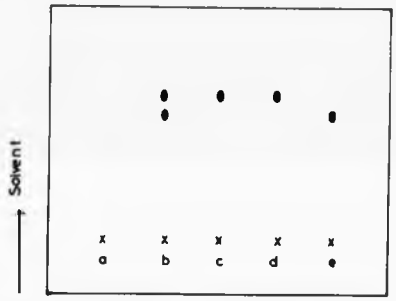


Fig. 5.5 Silica gel chromatogram showing the C-terminal residues cleaved by carboxypeptidase and controls. (a = normal HSA without carboxypeptidase; b = normal HSA with carboxypeptidase; c = Albumin Mundey with carboxypeptidase; d = L-leucine; e = L-glycine)

Therefore, the N-terminal residue of albumin Mundey is L-aspartic acid while L-leucine is C-terminal.

Analysis of the fragmentation products of Albumin Mundey by the methionine-specific and tryptophan-specific reagents are discussed separately in the next chapter.

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CHAPTER VI

STAGE 4: PEPTIDE MAPPING

This chapter deals with methods used to examine the products of chemical fragmentation of Albumin Mundey. A method for the preparative isolation of the mutant peptides is also described and the binding of bilirubin to Albumin Mundey studied by the fluorescence-quenching method.

CNBr fragments were analysed on 12% polyacrylamide gel containing 5 M urea, 6 mM Triton X-100 and 5% acetic acid[20]. This procedure was found to have a high degree of reproducibility and sensitivity to small changes in ionic charge of the peptides. This charge, which determines the electrophoretic mobility of the peptide, is due to differential micelle formation of this detergent with the non-polar regions of the peptide. These regions of the peptide are generally of high helical content[19]. Unlike most conventional gel systems, it is also able to resolve neutral amino acid substitutions. When long gels (48 cm) were used, a higher degree of resolution and clarity of the peptide maps were obtained as opposed to conventional gels of length 16 cm.

Fragmentation products of the tryptophan-specific cleavages were resolved on SDS-PAGE[8,16] or by SDS-gradient PAGE[12] (12-25%). However, no distinctive advantage was achieved by the use of the latter gel system.

In the initial stages of peptide mapping a highly sensitive silver stain[11,18] was used to detect peptides

that were below the sensitivity of the organic stain, Coomassie Blue. This stain is 100-fold more sensitive than Coomassie Blue. Protein bands are detected by a process analogous to the photographic printing process whereby ionic silver is reduced to the metallic form. However, it was found to be too sensitive to be of practical use as numerous artefactual bands were produced.

6.1 PREPARATIVE ISOLATION OF PEPTIDES

The same polyacrylamide gel system as in peptide mapping was used in the preparative isolation of the peptides. The peptides were extracted from the excised gel slices by reverse polarity electrophoretic elution[10] or by free diffusion from homogenised gels[5]. The identities of the peptides were established by use of dansyl chloride to label the N-terminal residue[15] and by molecular weight determination[8].

This preparative method, which entailed the excision of the peptide-containing gel immediately after rapid staining with Coomassie, was chosen against other methods such as chromatography for the following reasons.

(i) Positive identification of the mutant peptides was guaranteed.

(ii) The expected difficulty in identification of the mutant peptides by chromatographic fractionation as they are physiochemically very similar to their normal analogues.

(iii) Amino acid modifications occur in the peptides fractionated by chromatography.

This can produce problems in the ensuing sequence study. These modifications (a-d) are:

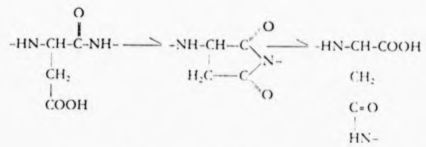
(a) the rearrangement of aspartic acid residues,

(b) the hydrolysis of asparagine and glutamine,

(c) oxidation of cysteinyl residues, and

(d) the conversion of N-terminal S-carboxymethylcysteine residues.

(a) Rearrangement of aspartyl residues involve α → β interconversion followed by deamidation. This results in the production of three different forms of this residue[13] (Scheme 6.1). Their multiple forms complicate sequence studies, especially in the Edman degradation (Section 7.1.2) where the imide or β-aspartyl structure blocks the cyclisation stage[13].

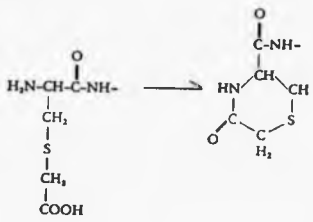


Scheme 6.1

(b) Both asparagine and glutamine are hydrolysed to aspartic and glutamic acids respectively.

(c) Sulphydryl groups in cysteines are easily oxidised to the sulphoxides.

(d) N-terminal S-carboxymethylcysteine can be converted to a thiazane carboxyl residue (Scheme 6.2).



Scheme 6.2

These reasons justified the preparative method used.

The extracted peptides were identified by determination of their N-terminal residue by dansylation[15] and by molecular weight determination by SDS-PAGE[8].

The binding of bilirubin to Albumin Mundey was determined by fluorescence-quenching studies. Albumin, like most proteins, exhibit ultraviolet fluorescence which can be quenched by binding of ligands. This quenching only occurs with binding[2] and the binding of bilirubin causes such quenching[1]. Therefore, with this technique both the capacity and affinity of albumin for bilirubin could be determined.

6.2 OTHER PREPARATIVE TECHNIQUES

This section describes two methods commonly used in fractionation of peptides. They are chromatography and HPLC.

6.2.1 Chromatography

Chromatographic separation of peptides are usually achieved by a combination of several techniques. These

are ion exchange chromatography, gel permeation chromatography and paper electrophoresis. In one variation, the mixture of peptides is initially fractionated on a cationic exchanger. The resulting impure fractions are rechromatographed on an anionic exchanger. Finally, the remaining unresolved peptides can then be fractionated by gel filtration or paper electrophoresis. Alternatively, the sequence of the above processes can be reversed, i.e. an initial resolution by gel filtration followed by fractionation by ion-exchange chromatography.

6.2.2 HPLC

Fullmer and Wasserman[3] described the use of a combination of solvents coupled with gradient elution from reversed phase support media in analytical and preparative peptide mapping. The solvent system was orthophosphoric acid-acetonitrile. Wilson[17], in a recent series of papers described the implementation of HPLC in the fractionation of peptides. Acetonitrile[4,6] and the hydrophilic ion-pairing solvent, orthophosphoric acid[6], were also used in the rapid and effective separation of peptides.

6.3 EXPERIMENTAL

6.3.1 Peptide Mapping[20]

The peptides were resolved on polyacrylamide gel (12%) containing the gel buffer, 5 M urea/6 mM Triton-X100

in 5% acetic acid. The initial fractionation was on short vertical slab gels (17 cm) but the resolution was vastly improved on gels of longer lengths (48 cm).

The gel was initially subjected to pre-electrophoresis at 360 V for 36 h after which constant wattage was obtained. It was then overlayered with a solution of the above gel buffer containing a reducing agent, dithiothreitol (0.5 M) to maintain the gel in a reduced state. This precaution reduced or prevented the oxidation of amino acids as described in Section 6.1. Similarly, all glassware was washed in thiodiglycol (0.1%) and all buffers used contained this agent in similar concentrations. Electrophoresis was carried out at 300 V until the Fuchsin Red marker was eluted out of the gel.

After electrophoresis the peptides were fixed in 10% TCA, stained in 0.1% Coomassie Blue in destaining solution, acetic acid/methanol/water 10:45:35 (v/v/v). The gel was then destained at room temperature.

6.3.2 Preparative isolation of peptides

The preparative gel, which was of similar composition to the analytical gel, was of dimensions 48 x 23 x 3 cm. Immediately after electrophoresis, the gel was rapidly stained (about three-quarters to one hour) in Coomassie Blue (0.2% w/v) dissolved in water. This process avoided amino acid modifications that could be acquired during prolonged staining in conventional solutions containing organic solvents. The lightly-stained

gel was then rinsed in water.

In the method of Gibson and Gracey[5], the peptide band was excised from the gel. It was finely homogenised in formic acid (60%) to form a gel/supernation suspension of proportions 1:1 (v/v). Peptides were eluted from the gel by magnetic stirring for 6 h at 4°C. The supernatant obtained by centrifugation was then removed. The homogenised gel was washed twice with formic acid (60%) to increase recovery of the gel. All the supernatants were combined and lyophilised.

To extract the dye, equal volumes of HCl (5.7 M) and *n*-octanol were added to the lyophilised peptide. After thorough mixing, the solutions were centrifuged and the dark blue organic layer removed. This extraction was repeated twice. The final acid phase was diluted with water and the formic acid removed by lyophilisation, resulting in pure peptides.

The electrophoretic elution of peptides by the method of Mendel-Hartig[10] was found to be more effective with SDS-PAGE[8,16] than with Triton-urea gels[20].

6.3.3 N-terminal determination of peptides

Modification of the method by Tamura[15]. The peptides (1 nmol) were dissolved in cold triethylamine (0.1 M, 10 µl). Cold dansyl chloride (0.5% w/v, 10 µl) in dry acetone was added and the reactants refrigerated for 16 h. The solvents were then removed at 40°C *in vacuo*.

Hydrochloric acid (5.7 M, 50 µl) was added and hydrolysis carried out in flame-sealed tubes for 18 h at 105°C. After drying again *in vacuo* at 40°C, the dansyl amino acid was identified by two dimentional TLC on micro-polyamide plates using the solvent system of Hartley[7].

6.3.4 Gradient gel[12]

A gradient gel was cast by magnetically mixing two polyacrylamide solutions of different cross-linkings and then introducing this polymerisation solution into the polymerising chamber at a steady rate (about 10 ml/min).

This gradient gel was *tris*-HCl (pH 8.8, 36.6% w/v) containing SDS (0.1% w/v) in polyacrylamide (12-25%). The electrolyte was *tris*-glycine [pH 8.3, *trisma* base (30.2 g) and glycine (144 g) dissolved to 1 litre water]. The stacking gel was *tris*-HCl (pH 6.8, 6%) in polyacrylamide (10%).

After electrophoresis, for about 7 h, the gel was stained overnight in Coomassie Blue R-250 (0.02%) in acetic acid (7%). The gel was then destained in methanol/acetic acid/water 5:2:13 (v/v/v).

6.3.5 Laemmli gel

The method was modified from Laemli[8]. The glass tube was pretreated with dimethyldichlorosilane to prevent adhesion of the gel to the tube and so ease removal

of the gel after electrophoresis. The gel (0.6 x 15 cm) was *tris*-HCl (pH 8.8, 0.375 M) in polyacrylamide (12.6%). The electrophoresis buffer was *tris* (0.5 M)/glycine (0.384 M) (pH 8.3). The gel and electrophoresis buffer both contained SDS (0.1% w/v and 1% w/v respectively).

Electrophoresis was carried out at 120 V, after which, the gel was fixed in isopropanol/acetic acid/water 5:2:13 (v/v/v). The stain was Coomassie Blue R-250 (0.1%) in methanol/acetic acid/water 5:2:13 (v/v/v). Destaining was also in this solution without the added dye.

The standard markers used were of molecular weight 2,512-16,949 and 12,300-78,000 obtained from BDH.

6.3.6 Silver stain[14]

The peptides in the gel were fixed in methanol/acetic acid/water 25:6:19 (v/v/v) for 1 h, followed by rinsing in ethanol/acetic acid/water 2:1:17 (v/v/v) for 20 min. The gel was rinsed again in water (1 litre) for 20 min., glutaraldehyde (1., 500 ml), followed by three rinses in water (1 litre) for 20 min each.

A freshly made silver stain was made by adding silver nitrate (20 ml, 3.88 g silver nitrate in 20 ml water) slowly but with rapid mixing to this solution (95 ml water, 9.2 ml 1 M NaOH and 7.0 ml 35% NH_4OH). Water was then added to the resulting concoction until the 500 ml mark. After staining the gel in this solution for 20 min., the gel was washed in water (1 litre) for 15 min. The silver ions were reduced in a Switzer Reducer (100 ml

ethanol, 60 mg citric acid and 0.25 ml 37% formaldehyde made up to 1 litre water). The final stages consisted of washing the gel in three washes of water (1 litre).

A number of precautions had to be observed with this staining process. These are

(i) all vessels must be very clean,

(ii) the gel must be agitated intermittently during all steps,

(iii) the gel must be handled with gloves since fingerprints are also stained!! and

(iv) the gel must be removed immediately from the Switzer Reducer when the bands are stained. Any prolonged immersion will result in very dark backgrounds. This background can, however, be decreased with commercial photographic fixer (1:9 v/v).

6.3.7 Bilirubin binding[9]

The binding of bilirubin was studied by the fluorescence-quenching method. The fluorescence of albumin (1.04 µmol/lit, 3 ml, pH 7.4) in a far UV-quartz cuvette was measured by adding portions of bilirubin (20 µmol/lit). After mixing, the fluorescence was measured using a Perkin-Elmer Fluorescence Spectrophotometer MPF-3 where the excitation filter had a maximal transmission at 294 nm with a half band width of 13 nm, while the emission maximum was 343 nm with half band width of 12 nm.

6.4 RESULTS AND DISCUSSION

Non-specific cleavages at tryptophan-214 by NBS, NCS and *o*-iodosobenzoic acid (Table 6.1) were attested by examination of the fragmentation products on SDS-PAGE[6]. Highly specific cleavage at this residue should produce three peptides. These are the uncleaved albumin, N-terminal fragment (residue 1-214) and the C-terminal fragment (residue 215-585).

Table 6.1 Fragments obtained by the cleavage of *o*-iodosobenzoic acid on albumins

Sample	*o*-Iodosobenzoic acid	R.M.* of protein band	Identity of protein band
Normal HSA	X	0.25	Uncleaved HSA
Normal HSA	✓	0.25	Uncleaved HSA
		0.29	†
		0.35	Unknown
		0.44	‡
Double albumins	X	0.26	Uncleaved albumins
Double albumins	✓	0.25	Uncleaved albumins
		0.30	†
		0.37	Unknown
		0.42	Unknown
		0.45	‡

R.M.* = relative mobility

$$= \frac{\text{distance migrated by protein}}{\text{distance migrated by tracking dye}} \times \frac{\text{length of gel before staining}}{\text{length of gel after staining}}$$

† = C-terminal fragment (residue 215-585)

‡ = N-terminal fragment (residue 1-214)

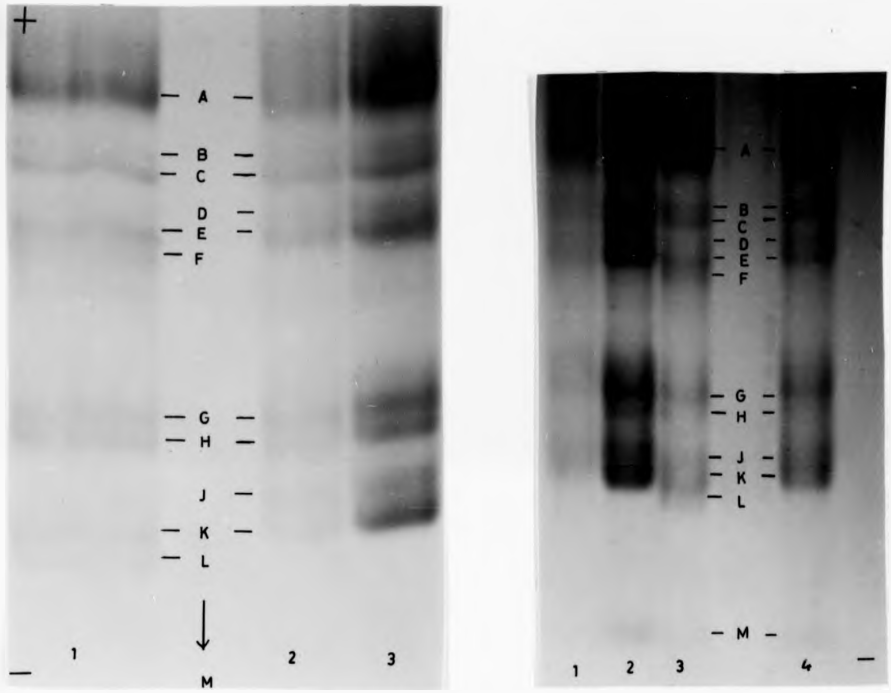
The results on Table 6.1 show that *o*-iodosobenzoic acid released an extra fragment from normal HSA while under identical experimental conditions, two extra fragments were

released. The identities of these extra fragments were not pursued further since the objective of this study was only to test the specificity of this reagent.

Examination of the fragmentation products of NBS and NCS cleavage also deviated from the expected quantity. Inconsistent numbers of extra fragments were produced under similar experimental conditions and it was difficult to characterise a typical cleavage. The extra fragments may arise from non-specific cleavages at tyrosyl and histidyl residues as described in Section 5.6.1.

On the contrary, the fragmentation patterns of CNBr cleavage, shown on Fig. 6.1 (a) and (b), were highly reproducible. They show the presence of two faster-migrating peptides, labelled (F) and (L), in Albumin Mundey that were not found in the control, normal HSA. Another observation is that each of the fragments (A-L) is present as two bands. The different form of the fragment is probably due to oxidation of the unreacted cysteinyl residues during reduction and alkylation as described in Section 5.1.

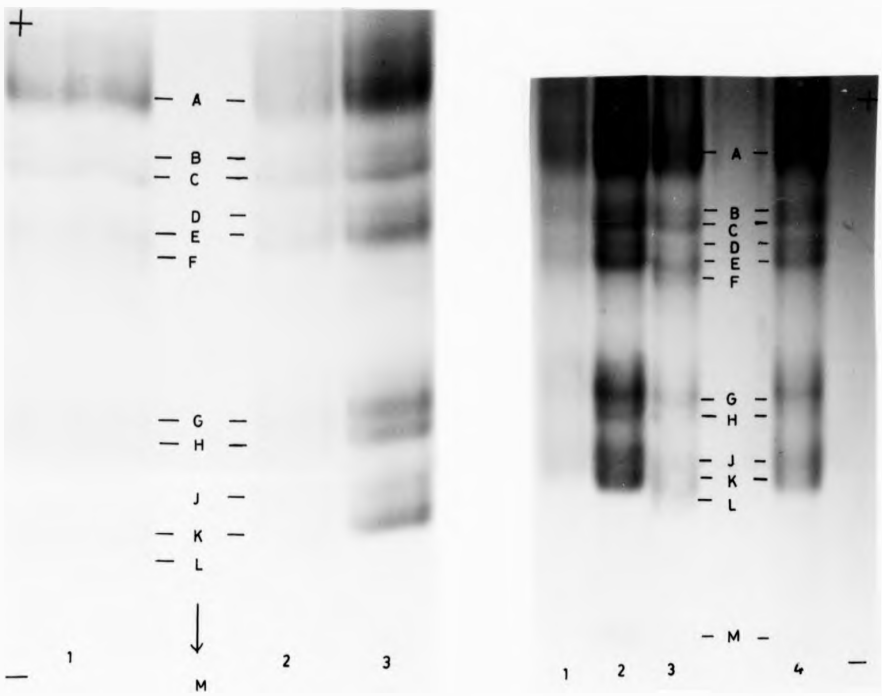
The eluted peptides were identified by N-terminal and molecular weight determination. Fig. 6.3(a) shows the dansylated N-terminals of the unseparated CNBr fragments of Albumin Mundey. The detected residues are L-aspartic acid, L-cysteine, L-alanine, L-proline and L-phenylalanine. It shows that the partially resistant bond at methionine-123 was cleaved since L-cysteine was detected. Only one cysteinyl residue (cysteine-214) was expected, as explained on Table 5.1. A quantitative estimation of this cleavage was unnecessary.



(a) (b)

Fig. 6.1 Resolution of CNBr fragments of isolated albumins
Track (1) Albumin Mundey Tracks (1,2 and 4) Normal HSA
Tracks (2,3) Normal HSA Track (3) Albumin Mundey
Length of gel = 48 cm.

Fig. 6.3(b) shows the N-terminal residues of the extracted CNBr peptides of Albumin Mundey. The same five residues, as in the unseparated fragments [Fig. 6.1(a)], were observed. However, it was relatively more difficult to interpret this chromatogram, as the mobility of these residues were altered by the polarity of the solvents used in the extraction process. Therefore, they were identified by their relative positions to each other on the chromatograms rather than with the standard dansyl-L-amino



(a) (b)

Fig. 6.1 Resolution of CNBr fragments of isolated albumins
Track (1) Albumin Mundey Tracks (1,2 and 4) Normal HSA
Tracks (2,3) Normal HSA Track (3) Albumin Mundey
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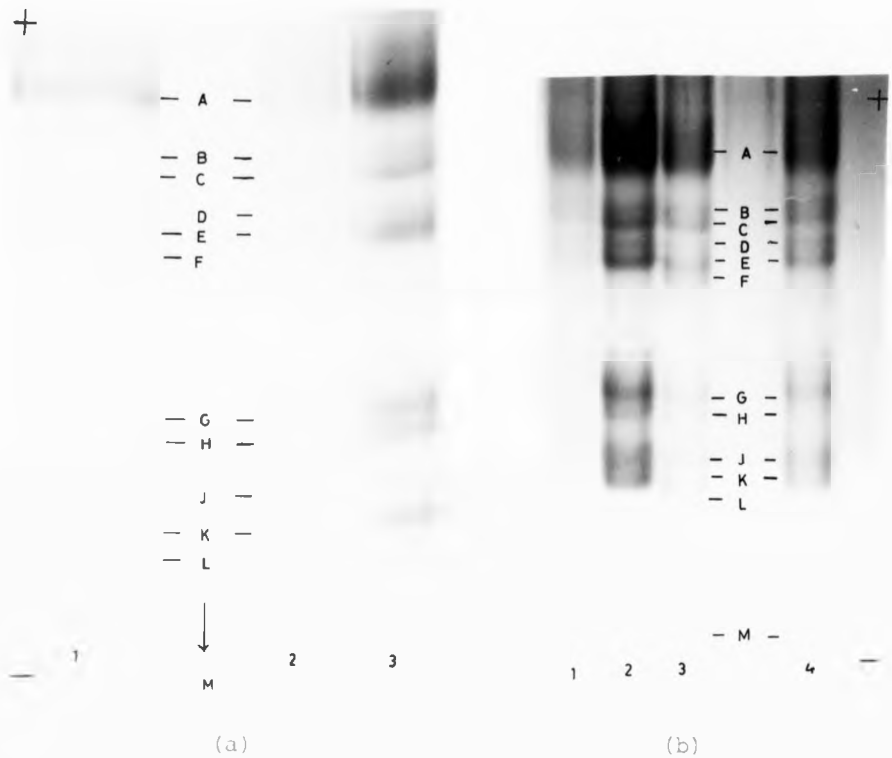

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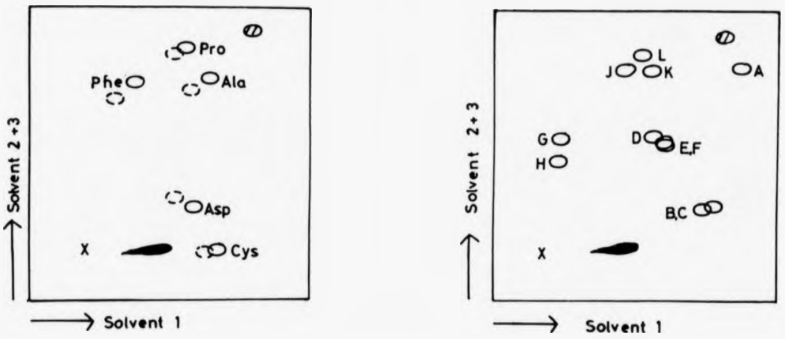

(a) (b)

Fig. 6.2 Chromatograms of DNS-amino acids on micro-polyamide plates

(a) CNBr fragments of Albumin Mundey
O = standard amino acids
O = dansylated amino acids of Albumin Mundey fragments
Ø = DNS-NH_2
● = DNS-OH

(b) Isolated CNBr fragment of albumin Mundey

CNBr fragments
Ø = DNS-NH_2
● = DNS-OH

acids, which were not exposed to these solvents. A further positive identification was made by molecular weight determination on SDS-PAGE. These results are summarised on Table 6.2.

In conclusion, the two mutant peptides (F and L) in Albumin Mundey correspond to CNBr fragments I (res. 1-87) and VI (res. 447-548).

Since one of the sites of mutation in Albumin Mundey is between residues 447-548, defective binding of bilirubin and palmitate are expected. The secondary binding site of bilirubin is between residues 446-547 (Section 1.5.2.3) and the primary binding site for palmitate is between residues 377-582. Also the mutation in the N-terminal (between residues 1-87) may impair

Table 6.2 The CNBr peptides of Albumin Mundey

Peptide	N-terminal residue	Approximate mol. wt. 10^3daltons	Residue number	CNBr fragment number
A	ala	4,21	88-123,88-298	II,II+III
B	cys	18	18	III
C	cys	18	124-298	III
D	asp	9	1-87	I
E	asp	9	1-87	I
F	asp	9	1-87	I
G	phe	12	330-446	V
H	phe	12	330-446	V
J	pro	11	447-548	VI
K	pro	11	447-548	VI
L	pro	11	447-548	VI
M	ND	ND	ND	

ND = not determined

binding of copper and nickel (Section 1.5.4.2).

A preliminary study using the fluorescence-quenching technique to study the binding of bilirubin to albumin shows that Albumin Mundey has both a slight decreased capacity and affinity for this ligand.

Fig. 6.2 shows a comparison of the fluorescence-quenching of Albumin Mundey and the control, normal HSA. It shows that the bilirubin binding site of Albumin Mundey is saturated at a slightly lower concentration than normal albumin and threfore has a decreased affinity for bilirubin.

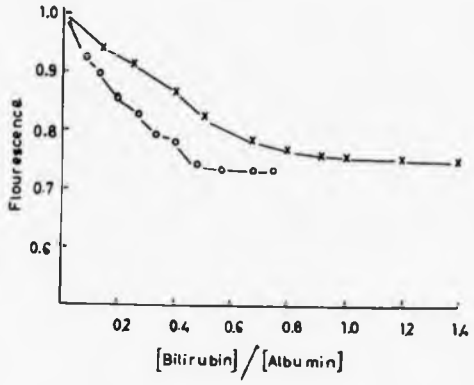

Fig. 6.2 Fluorescence-quenching titration of albumin, 1.64 µmol/lit with bilirubin
X = normal HSA O = Albumin Mundey

Since the sites of mutation on Albumin Mundey are now known, further studies on the binding studies of Albumin Mundey should include the estimation of binding affinity (K_A) of this ligand; palmitate, possibly by use of ^{3}H-palmitate and of copper and nickel.

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CHAPTER VII

STAGE 5: AMINO ACID ANALYSIS OF THE MUTANT PEPTIDE

This was the last experimental stage in quest of identification of the mutant amino acids in Albumin Mundey. It was unfortunate that this objective was not fulfilled due to insufficient time. However, in this chapter, some classical peptide/protein sequencing techniques are contrasted. These are the well known Edman degradation reaction, column chromatography and tryptic digestion. Operations of the Edman degradation in the manual, automated, solid phase and microsequencing mode are described. The phenylisothiocyanate amino acids, produced by this degradation, are analysed by high pressure liquid chromatography (HPLC), mass spectrometry and gas-liquid chromatography (GLC).

7.1 EDMAN DEGRADATION

This is a classical technique for determining the primary structure of proteins and peptides. It was formulated over 25 years ago and still remains a popular method. In this reaction[15], successive N-terminal amino acid residues of the protein or peptide react with phenylisothiocyanate* to form the phenylthiohydantoin† - amino acids. Each degradation step consists of three operations: coupling, cleavage and conversion.

---

*Phenylisothiocyanate = PITC
†Phenylthiohydantoin = PTH

7.1.1 Coupling[14]

In this reaction the free α-amino group of the N-terminal amino acid couples with phenylisothiocyanate to form the phenylthiocarbamyl[‡]-peptide (Scheme 7.1).

$$C_6H_5 \cdot NCS + NH_2 \cdot CHR \cdot CO \cdot NH \cdot X \rightarrow C_6H_5 \cdot NH \cdot CS \cdot NH \cdot CHR \cdot CO \cdot NH \cdot X$$

Scheme 7.1

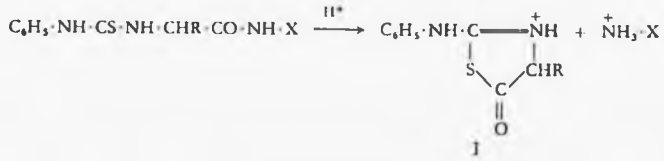
The rate of reaction is dependent on the *pK* of the amino group. The usual reaction conditions are pH 9 and temperature 40°C.

A side reaction that can occur is the oxidative desulphuration of the phenylthiocarbamyl group. This prevents the formation of the thiazolinone and so blocks any further degradation[35].

7.1.2 Cleavage[20]

The peptide bond nearest the PTC-substituent is rapidly cleaved in a strong acidic medium to produce the 2-anilino-5-thiazolinone derivative (I) (Scheme 7.2). The new peptide is shorter by one amino acid residue and the free α-amino group can undergo another cycle of degradation.

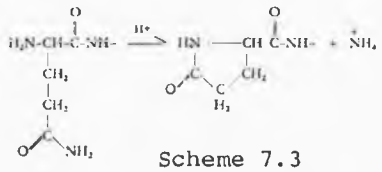
[‡]Phenylthiocarbamyl = PTC



Scheme 7.2

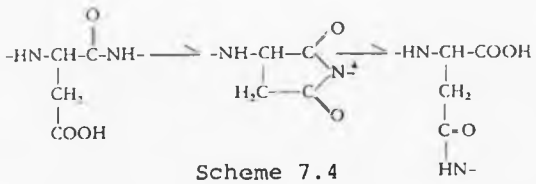
Under severe experimental conditions some side-reactions can occur.

(i) An N-terminal glutamic acid can cyclise to form a pyroglutamic acid (Scheme 7.3). This stops any further degradation[61] by blocking the α-amino group.



Scheme 7.3

(ii) The α → β interconversion of aspartyl residues and asparagine residues[61], can occur even under mild conditions. The β-aspartyl bond is not cleaved so further degradation is halted (Scheme 7.4).



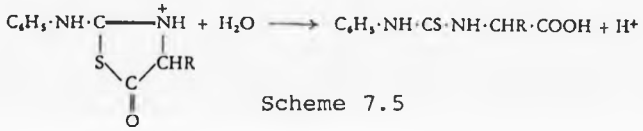
Scheme 7.4

(iii) When the degradation involves a histidine group, both this residue and the one adjacent to it are simultaneously released[66].

7.1.3 Conversion

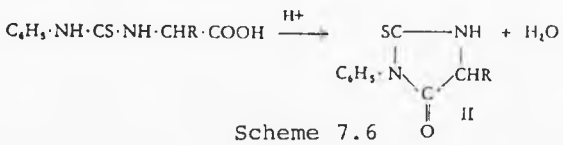
Conversion of the unstable thiazolinone derivative to the isomeric stable 3-phenyl-2-thiohydantoin (II) actually consists of two steps, hydrolysis and cyclisation.

The first step (Scheme 7.5) is the hydrolysis of the thiazolinone to the PTC-amino acid. This reaction



Scheme 7.5

in acidic aqueous medium is much faster than the second one (Scheme 7.6), where the PTC-amino acid cyclises to form the corresponding PTH-amino acid[3].



Scheme 7.6

Some side reactions can easily occur at this stage. These are (i) the decomposition of PTH-serine by β-elimination which is followed by a possible polymerisation reaction[17]; (ii) partial hydrolysis of the amide groups of PTH-asparagine and PTH-glutamine. Their respective PTH-aspartic acid and PTH-glutamic acid are formed; (iii) desulphurisation of the phenylthiocarbamyl group by oxidation[35] which also can occur in the coupling reaction (Section 7.1.1).

7.2 MODES OF OPERATION

7.2.1 Automated Edman Degradation

The invention of the automated protein sequenator by Edman and Begg[16] revolutionised the field of protein sequencing. This instrument only performs the coupling and cleavage reactions. The conversion reaction is left to conventional methods. The two steps are completed in about 95 min. Therefore 15 cycles can be achieved in a day compared to one cycle by manual methods.

The principle of the sequenator is that intact proteins or long peptides are repetitively sequenced (coupling and cleavage) inside a spinning cylindrical glass cup. The proteins/peptides react with phenylisothiocyanate (in n-hexane or heptane) and the coupling medium (Quadrol - trifluoroacetic acid (pH 9.0). These reagents form a thin spinning film. By sliding another thin film of benzene and ethylacetate, excess PITC, Quadrol and side products are removed. In the cleavage reaction, a short exposure of the PTC-peptide to anhydrous heptafluorobutyric acid forms anilinothiazolinone. The thiazolinone is then extracted with 1-chlorobutane.

The yield of this repetitive operation is about 98%. Losses in material occur during washings in the last stages of degradation. The shortened peptide is easily removed together with the thiazolinone during cleavage.

Some steps to minimise losses are: (i) not

repeating the cleavage process and (ii) the use of Braunitzer reagents[4,5] (4-sulphobenzeneisothiocyanate, 3,5-disulphobenzeneisothiocyanate and 4,6,8-trisulphonaphthalene-2-isothiocyanate). These highly polar reagents couple with the lysine and S-aminoethyl cysteine residues of a peptide. Hydrophilic moieties are formed with reduced solubility in organic solvents.

7.2.2 Solid-phase Edman Degradation

This is the repetitive isothiocyanate degradation of short peptides anchored to insoluble supports[38,39] such as resins, polyamides[8], polystyrenes[38] or glass[12]. This technique is ideal for sequence determination of CNBr fragments[32] and tryptic peptides with lysine or ornithine as C-terminal residues[39]. The advantages of solid phase degradations are that peptide losses through extractions are minimised (contrast Section 7.2.1) and that the coupling and cleavage processes can be accomplished simply by elution of the reaction buffers into the column.

Most of the problems associated with solid-phase degradation are concerned with support methods and increasing the yield of peptides attached to solid supports. Ideally, the C-terminal carboxy group on the bridgehead of the peptide should be attached to the insoluble support.

Edman degradation can then proceed from the unattached N-terminal end. However, this is not always possible, especially with carboxyl groups of glutamic and aspartic acids. This is due to similar reactivities of their C-terminal carboxyl groups. However, selective

attachment of the aspartyl residue can apparently be achieved by modification of the carboxyl group activation procedure[52].

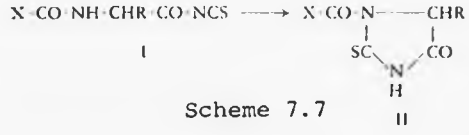
A variation of solid-phase degradation is carboxyl-terminal sequencing. It uses di-*p*-nitrophenyl-phosphoryl azide to form an acyl azide with the peptide carboxyl terminus. The carboxyl terminus is released as an aldehyde derivative by thermolysis of the acyl azide[46], to form a peptide-amide. The product formed by reaction of the peptide amide with 1,1-*bis*(trifluoroacetoxy)iodobenzene produces an aldehyde and a new peptide amide[47].

In the reverse principle of the solid support, the isothiocyanate groups are attached to the support instead of the peptide[13].

7.2.3 Manual Edman Degradation

This manual degradation of a peptide can be from the conventional N-terminal end (Section 7.3.1) or from the C-terminal[57].

In degradation from the C-terminal end, the thiocyanate reacts with the C-terminal residue of the peptide (Scheme 7.7). The acyl isothiocyanate (I) spontaneously rearranges to the 1-acyl-2-thiohydantoin derivative (II).



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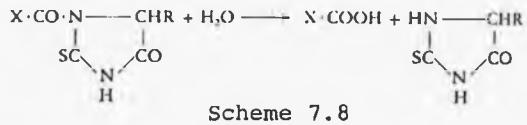
Scheme 7.7

The acyl group is then hydrolysed to form 2-thiohydantoin (Scheme 7.8) which creates a new C-terminal available for

another degradation cycle.



Scheme 7.8

Analysis of amino acids released by the Edman degradation as their highly sensitive dansyl derivatives were pioneered by Gray and Hartley[23]. Repetitive analysis can be 'subtractive' where the cleaved N-terminal residue is identified after the cleavage and conversion steps[30], or by hydrolysis of the PTH-derivatives[59] to regenerate the amino acids.

7.2.4 Microsequencing

Chang *et al.*[9] reports the microsequencing of a peptide by a double coupling method using organic isothiocyanates. The first coupling is with 4-N,N-dimethylamino-azobenzene-4'-isothiocyanate and the second with the Edman reagent, phenylisothiocyanate. The resulting coloured thiohydantoins are easily detectable on chromatograms.

7.3 ANALYSIS OF PTH-AMINO ACIDS

PTH-amino acids may be separated and identified by chromatographic methods such as paper[58], thin layer[10], gas-liquid (GLC)[51], high pressure liquid chromatography (HPLC) or by mass spectrometry (MS).

In all these systems the PTH-amino acids can be identified by a direct or an indirect method. The direct method relies on the positive identification of the released moiety[19,54,65]. In the indirect method, identification depends on a difference in analysis of the peptide before and after a degradation step.

Two techniques of the indirect method exist. In the method of Hirs *et al.*[30], quantitative amino acid analysis of the peptide is made before and after a degradation cycle. In the method of Gray and Hartley[23], only the N-terminal amino acid is determined before and after a degradation cycle. This latter technique is less ambiguous than the former method.

In the following sections, analysis of PTH-amino acids by HPLC, GLC and MS are discussed.

7.3.1 High Performance Liquid Chromatography (HPLC)

The separation of PTH-amino acid by HPLC is usually by adsorption chromatography. Here the mobile phase is liquid and the stationary phase is solid. The solid particles are very fine (3-10 μ in diameter). The two modes of adsorption chromatography to accomplish separation can be ordinary adsorption or reverse-phase chromatography.

Ordinary adsorption chromatography has a polar stationary phase (e.g. silica gel, porous glass beads or alumina) and the mobile phase is relatively non-polar (e.g. hexane or chloroform[29]). Therefore, PTH-amino acids

have to compete with the mobile liquid phase for adsorption. Their retention onto the stationary phase is dependent on their basicity. The order of elution of these moieties is such that non-polar substances are followed by those of increasing polarity. The last group of PTH-amino acids, highly charged derivatives, is very difficult to elute because of strong adhesion. Therefore, reverse phase chromatography coupled to gradient elution or to ion pair chromatography is advisable.

Ion pair chromatography is a special form of liquid-liquid chromatography (liquid stationary and mobile phases) which, when used in the reverse-phase mode with a hydrocarbon bonded stationary phase, can be used for the separation of polar amino acid derivatives.

One of the explanations for their retention onto the hydrophobic stationary phase is that the ionic amino acid forms an ion pair with a suitable counter-ion in the mobile phase that increase its lipophilic character[37].

In reverse phase chromatography, the stationary phase is non-polar (e.g. polymer beads) but the mobile phase is polar (e.g. water or ethanol[29]). The order of elution of PTH-amino acids is the reverse from that in ordinary chromatography. The polar derivatives are eluted first followed by those of decreasing polarity. Recently, Black and Coon[2] reported the separation of PTH-amino acids with wide polarity differences by the application of a step-gradient.

The choice of either ordinary adsorption or reverse phase chromatography is, as stated, dependent on

the chemical character of the heterogenous pool of PTH-amino acids. These chemical characteristics can be divided into three classes: non-polar, relatively polar or highly polar[6]. The non-polar PTH-amino acids (pro, leu, ile, val, phe, met and ala) can be separated under mild lipophilic solvent conditions on highly efficient columns, since their common structural differences are in the length and branching of their aliphatic side chains. The second group, relatively polar derivatives, try, gly, lys, tyr, thr, ser, asn and gln), must be separated by gradient elution because of the wide variety in polarity of their functional groups.

The last group of highly charged acids and bases (glu, asp, cyn, his and arg) poses the largest problem in separation and identification due to their strong adhesion to the stationary phase. Ion pair chromatography or adsorption chromatographycoupled to reverse phase chromatography may solve this problem.

7.3.2 Gas-liquid Chromatography (GLC)

The PTH-amino acids are very diverse in their chemical and chromatographic properties, with respect to GLC. They can be classed into three groups, volatile, less volatile and involatile[50].

Group I PTH-derivatives (ala, gly, val, leu, ile, met, pro, phe) are the most volatile and their presence are recorded as symmetrical peaks. Group II derivatives (gln, asn, tyr, his, and tryptophan) are less volatile. All,

except tryptophan, adsorb strongly onto the chromatographic particles. They are recorded as trailing peaks. Group III derivatives are unstable and involatile (asp, glu, lys, ser, thr, cys) and must first be converted to their volatile trimethylsilyl derivatives before analysis[24,48].

The general procedure in gas-liquid chromatography is that PTH-amino acids are first volatised or converted to their more volatile form by, for example, silylation. They are then introduced into the stationary liquid phase of a high melting polymer-coated particle (e.g. chromasorb W) packed in a long glass column (PTH-amino acids are destroyed by contact with metals)[33]. They are then heated at an optimised temperature ranging from 160-290^{o}C. An inert gas, helium[49], or nitrogen, is used as the moving phase and the vapourised derivatives partition themselves between the stationary liquid phase and moving gas phase according to their individual gas-liquid coefficients. The current generated by the flow of the ionised fragments of the PTH-derivatives in a hydrogen flame defector (300^{o}C) is monitored and recorded as separate peaks.

7.3.3 Mass Spectrometry (MS)

This is another valuable method for analysis of PTH-amino acids[19,42,55]. The chemical ionisation method (CI) is usually preferred over the more common electron ionisation (EI) mode since it is more sensitive and spectral bands are fewer which eases their interpretation[21].

A unique feature of MS is that it is possible

to analyse samples after cleavage without prior conversion to the thiohydantoin[56]. This is because thiohydantoins are formed from a thiocarbamyl peptide[18] or a thiazoline[20] by the heat of the probe.

7.4 COLUMN CHROMATOGRAPHY

The principle involved in column chromatography is the resolution of a mixture of amino acids into individual amino acids on an ion exchange column consisting of very fine and homogenous particles. This technique is the prototype of HPLC.

Factors that determine the sensitivity, reproducibility and resolution are (i) sample preparation, (ii) particle size, homogeneity and degree of cross-linking in the ion exchange resin, (iii) ionic compositions, ionic strengths, pH and temperatures of the eluting buffer, (iv) flow rate and pressure of the eluting buffer and finally (v) dimensions (length and diameter) of the chromatographic column.

Examples of careful sample preparation are as follows: (i) Hydrolysis of the pure peptide under oxygen-free conditions to minimise destruction of amino acids by oxidation. The extent of amino acid destruction is also dependent on the character of the peptide, time and temperature of hydrolysis. The most labile amino acids are tryptophan, cysteine, cystine, serine, threonine and tyrosine[40]. (ii) Incomplete hydrolysis of some peptides can occur between resistant bonds, e.g. between sterically

hindered amino acids. (iii)Also,amino acids are released at different rates during hydrolysis. The result is that the total concentration of amino acids may not necessarily correlate with that of the original peptide.

Like all column chromatographic techniques, the sample volume should not be more than 10% of total column volume. The sample should have a low salt concentration and be free of contaminants such as air borne and particulate substances. Amino acids were found in air borne particles[53] and even finger prints[45].

The features of a good chromatographic column of high resolving power are that it should be properly packed with very fine[25] homogenous and uniformly cross-linked (usually 4-12%)[28] ion exchange resin.

The citrate buffer systems formulated by Spackman[62] in 1958 are used today even in amino acid analysers. Acidic and neutral amino acids are separated by citrate buffers of pH 3.25 and 4.25 respectively. The basic and very strongly adsorped amino acids are eluted with buffers of pH 5.25 and 10.1[62]. Delivery of the buffers is at a constant rate at high pressure[26,43].

Decreasing the column diameter increases the sensitivity proportionately. A decrease in the column length increases the speed and sensitivity but decreases the resolution[27]. The average column is of dimensions 0.9 x 150 cm[44], but a twenty-fold increase in sensitivity can be achieved on a 0.28 x 60 cm column. A microbore column of 0.05 cm diameter increases the sensitivity even further by 300 times[36].

The eluted amino acids are detected by reaction with ninhydrin[41] or fluorescamine[67]. Ninhydrin forms a purple colour with most amino acids or a yellow colour with proline or hydroxyproline. Fluorescamine reacts almost instantaneously with primary amines at pH 9 at room temperature. It forms a highly fluorescent product. However, proline and hydroxy proline must first be oxidised to primary amines by N-chlorosuccinimide before reaction with fluorescamine.

7.5 DIGESTION BY TRYPSIN

Treatment of the mutant peptide with trypsin, the most specific of the endopeptidases, result in cleavage at lysyl and arginyl residues. Under careful optimised conditions, the extent of fragmentation can be controlled.

Slow albumin mutants usually arise from basic to acidic amino acid substitutions (the other possibility being neutral to acidic substitutions). The involvement of a lysyl residue in this mutation can be proven by selectively modifying the lysyl residues in the mutant peptide by chemical means.

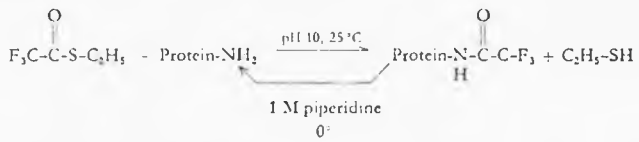
Analytical comparison of this digest against a corresponding normal peptide by finger printing methods on, for example, two dimensional Cleveland gels[11], should reveal a peptide map of the mutant moiety with one less peptide than the total in the corresponding normal peptide.

The sequence of the small fragment in the larger

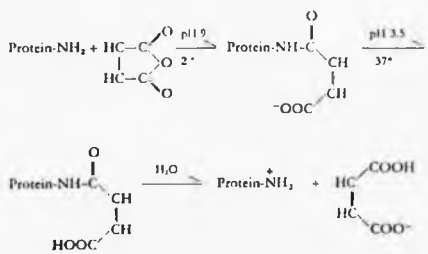
mutant peptide is deduced by realignment of the tryptic fragments. The identities of the peptides can be confirmed by determining their N-terminal and C-terminal residues. Since the mutant tryptic peptide is now sufficiently small, manual Edman degradation (Section 7.2.3) can successfully reveal its sequence.

The rates of tryptic cleavage of the susceptible bonds in this peptide are influenced by the chemical characters of the side chains of the amino acids in the susceptible bonds. Slower rates in hydrolysis occur when the susceptible bond is adjacent to an acidic amino acid[1] and consist of repetitive sequences of arginine and/or lysine. The imino nitrogen of proline renders the bond practically resistant to cleavage[60].

As mentioned, chemical modification of the ε-amino group of lysine restricts hydrolysis specifically at arginyl residues. Trifluoroacetylation[22] (Scheme 7.9) and maleylation[7] (Scheme 7.10) are the two most popular



Scheme 7.9



Scheme 7.10

methods because of the ease in their removal under mild conditions to regenerate the unmodified amine functions and also their high specificity for the α- and ε-amino groups of proteins.

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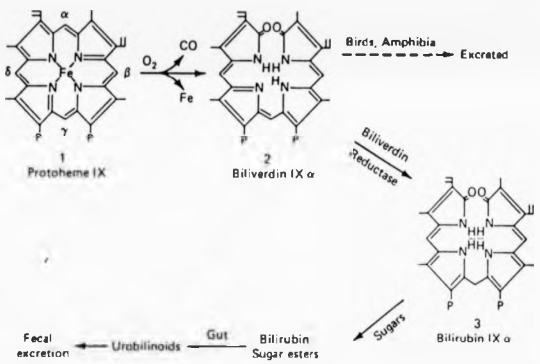
CHAPTER VIII

STUDY ON THE PHOTOINDUCED ISOMERISATION OF BILIRUBIN-IXα IN DETERGENTS

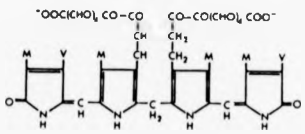
8.1 INTRODUCTION

The end product of haeme degradation (Scheme 8.1) is the linear tetrapyrrole, bilirubin-IXα (24). This



Scheme 8.1

functionless and toxic end product is removed from the circulation by an essential esterification of its two propionyl side chains with carbohydrates (eg, glucuronic acid glucose and xylose) to form mono- or diester. This reaction is catalysed by glucuronyl-, glycosyl- and xylosyl-transferases in the liver[7,12,29]. Bilirubin diglucuronide is shown on (23).



(23)

A failure in this detoxification system can lead to hyperbilirubinemia, which manifests itself by a yellow colouration of the skin. Even in early civilisation, Hippocrates expounded its prognosis and diagnosis[1] and the intricate chemistry of this waste product of haeme catabolism has continued to fuel the researches of photo-, analytical-, organic- and physio-chemists.

8.2 PHOTOTHERAPY OF NEO-NATAL JAUNDICE

The widely-used therapy for neo-natal jaundice is photo-irradiation of the skin with blue and white light from luminescent sources[27]. Maximum effect in phototherapy is experienced in light of approximate wavelength, 550 nm. This coincides with the bilirubin absorption maximum of 450-600 nm[28].

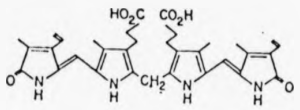
During phototherapy, the bilirubin concentration is decreased by both photochemical decomposition and elimination of undecomposed bilirubin[19].

The photochemical decomposition of bilirubin occurs in the surface layers of the skin and not in the vessels[2]. Furthermore, these oxidation products, which are absorbed by the skin by binding to collagen and lipids, are unlike those produced in aqueous solution[17].

The undecomposed bilirubin (mainly *cis-trans* isomers) is eliminated from the organism in the unbound state. This is unlike the conventional method of elimination as conjugated bilirubin[18]

8.3 STRUCTURE OF BILIRUBIN

Bilirubin-IXα (24) consists of four pyrrole rings



(24)

linked by three carbon bridges. The side chains consist of two vinyl, four methyls and two propionic acid moieties. This naturally occurring isomer is derived from the scission of the photoporphyrin IXα ring [Scheme 8.1(1)] at the α-carbon bridge. It exists predominantly in the lactam form (NH-C=O) rather than the corresponding tautomeric lactim (N=C-OH) form[14].

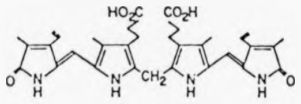
8.4 ISOMERS OF BILIRUBIN

Modification of the basic structure of bilirubin-IXα can give rise to structural and geometrical (*cis-trans*) isomers.

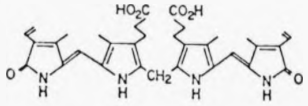
Structural isomers can be induced *in vitro* or produced *in vivo*. *In vitro* reversible isomerisation can be catalysed by strong non-oxidising acids[26], by aerobic incubation in the dark[24] or anaerobic irradiation with visible light.

The latter two mechanisms appear to be catalysed by a free radical process[21]. In all three cases, random recombination of the dipyrroles (formed by cleavage at

the central methylene bridge) results in a mixture of the bilirubin-XIIIα (25), -IXα and -IIIα (26) isomers in an approximate molar ratio of 1:2:1[25].



(25)



(26)

These isomers are also found in the serum of jaundiced new-borns where the IIIα and XIIIα isomers are in near equal proportion. Collectively, they constitute between 30-40% of the total bilirubin concentration[27].

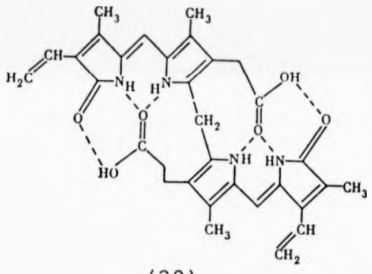
Geometrical isomers of bilirubin arise from the different positions (Z or E) of substituents relative to the two enamide C=C bond. The possible permutations, are shown below (27).

(27)

Naturally occurring bilirubin-IXα has a ridge-tile configuration (28). Rings A and B are coplanar and are at right angles to the coplanar rings, C and D. This contortion, due to the Z,Z configuration at C_4-C_5 and

(28)

C_{15}-C_{16}, is maintained by six strong intramolecular hydrogen bonds formed along the ridge of C_8-C_{12} (29).



(29)

These bonds are formed between the amide and carboxyl moieties of the carboxylic acid groups, pyrrole-imino hydrogens and the terminal lactam system[4].

8.5 CHEMICAL PROPERTIES

The chemical properties of bilirubin are predictable from its chemical structure. The central methylene bridge, which separates the dipyrrole segments, is especially prone to attack by electrophiles, oxidative dehydrogenation and cleavage[22]. However, the outer dipyrrylmethane bridges are more stable.

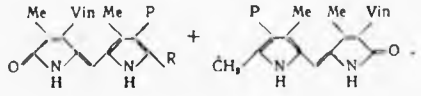
Bilirubin has two weakly acidic carboxylic acid side chains (propionic acid) which readily form esters. Esterification with alcohols renders the molecule susceptible to autoxidation since the central methylene bridge becomes exposed. This results in an unstable molecule[11]. Its acidic properties can also be increased in an alkaline medium by the tautomeric transition to the bislactim form.

The basicity of bilirubin is conferred by the nitrogen atoms of the central pyrrole rings. The double bonds on these rings are, however, more resistant to reduction than those in the outer rings and side chains.

8.6 PHOTOCHEMICAL ISOMERISATION OF BILIRUBIN

The photoisomerisation of bilirubin-IXα to its structural isomers, IIIα and XIIIα, occurs frequently[21] and is the cause of contamination in commercial bilirubin.

A proposed mechanism (Scheme 8.2) involves a free radical, R^{O}, which may be bilirubin or any component of this system. The free radical attacks bilirubin-IXα



Scheme 8.2

at C_9 or C_{11} to form tetrapyrrole free radicals. These then decompose into two dipyrrole fragments. In the following chain propagation reaction, the radical fragment

can attack an unreacted molecule of bilirubin at C_9 or C_{11}. A hexapyrrole free radical is then formed from which another free radical dipyrrole fragment is split off. The end result of this chain reaction is the production of bilirubin-IIIα and -XIIIα in place of the original -IXα molecule[27]. The absorption of light also converts bilirubin-IXα to a probable mixture of *cis-trans* (Z,E) geometric isomers about C_4-C_9 and C_{15}-C_{16} bonds in the tetrapyrroles.

8.7 AIMS OF THIS STUDY

Detergents greatly enhance the activity of 'native' glucuronyltransferase in hepatic microsomal membrane preparations (Section 8.9). The aim of this study was to investigate the possibility that detergent micelles may also catalyse the photo-isomerisation of bilirubin-IXα. These detergent-water systems are analogous to the behaviour of naturally occurring lipids[6], therefore, the ionic characters of the detergents used were chosen to mimic that of the phospholipids of membranes. Also, the disproportionation of bilirubin monoglucuronide into unconjugated bilirubin and bilirubin diglucuronide on photoirradiation in micelles was also studied.

The following sections discuss micellar catalysis and the two opposing theories on the conjugation of bilirubin prior to excretion.

8.8 MICELLAR CATALYSIS

In dilute solutions, detergents will specifically orientate to form high molecular weight aggregates or micelles. They consist of a hydrophobic core surrounded by a hydrophilic coat.

The narrow range at which micelles are formed is determined by the critical micellar concentration (CMC).

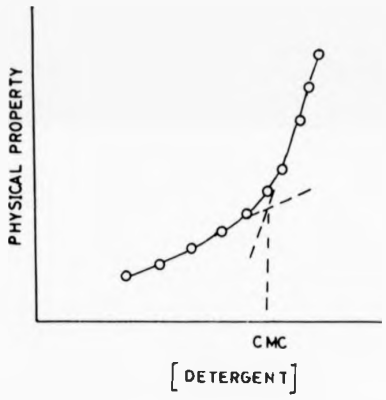


Fig. 8.1 Changes in a physical property as a function of detergent concentration.

This value for detergents with long aliphatic chain is usually between 10^{-4} and 10^{-2} M. For non-ionic micelles, this concentration is 100-fold smaller than for ionic micelles with comparable hydrophobic groups. The positive charge of anionic detergents is less exposed than the negative charge of anionic micelles and they are more compact in structure[9].

The cationic detergent, cetyltrimethylammonium bromide $[CH_3(CH_2)_{15}N^+(CH_3)_3Br^-]$ was used to mimic the

naturally occurring phosphoglyceride, phosphatidylcholine (lecithin) with $-CH_2-CH_2-N^+(CH_3)_3$ as a functional group. Also used were the neutral and anionic detergents, Tween 20 and sodium dodecyl sulphate.

8.9 CONJUGATION OF BILIRUBIN

In humans and most mammals, bilirubin is conjugated as the diglucuronide in a reaction catalysed by glucuronyltransferase. In hepatic microsomal membrane preparations, the activity of this enzyme is greatly enhanced by agents that agitate the membrane. They probably relax the constrains which an intact membrane imposes on the enzyme. These agents may be high pH, detergent, ultrasonication, several glucuronides or brief treatment with phospholipase A[5,8,13].

Formation of Bilirubin Diglucuronide

Bilirubin monoglucuronide (BMG) is formed from bilirubin-IXα in a well-established reaction, catalysed by UDP-glucuronosyltransferase. However, the ensuing mechanism resulting in formation of bilirubin diglucuronide (BDG) is subject to controversy.

Two different mechanisms have been proposed. The first mechanism, proposed by Heirwegh[13] is generally accepted, where the diglucuronide is formed by a sequential mechanism. The glucuronosyl residue is transferred from UDP-glucuronic acid to the unesterified propionic acid side-chain of BMG in a reaction catalysed by UDP-glucuronosyltransferase.

The second opposing mechanism, proposed by Jansen[15] is based on a transesterification reaction. This reaction involves the transfer of a glucuronosyl group from one BMG molecule to another (transglucuronidation), resulting in equimolar concentrations of BMG and unconjugated bilirubin. This mechanism differs from the former in several factors. These are:

(i) The enzyme is located in the liver plasma membrane instead of the microsomes.

(ii) The formation of BDG from BMG is catalysed by BMG- and not BDG-glucuronosyltransferase.

(iii) This transglucuronidation reaction does not require UDP-glucuronic acid as sugar donor.

8.10 EXPERIMENTAL

All operations involving bilirubin were carried out in subdued light.

8.10.1 Purification of bilirubin

The method of Fog[10] was used to remove contaminants from commercial bilirubin-IXα.

8.10.2 Photoisomerisation of bilirubin in detergent solution

All solutions used were thoroughly degassed before use. Bilirubin (2 mg, 3.33 mmol) was dissolved in dilute KOH (0.1 M, 9 ml) and phosphate buffer (0.1 M, 10 ml)

containing detergent (1 mM) was immediately added. Detergents used were cetyltrimethylammonium bromide (CTAB), sodium dodecyl sulphate and poly(oxyethylene) sorbitan mono-oleate (Tween 20). These respective detergents are cationic, anionic and neutral in character.

The solutions were then placed in test-tubes open to the air in a water bath at 20 ± 0.5°C and irradiated by a 60 W tungsten lamp placed perpendicularly 8 cm away from the tubes. At appropriate time intervals, portions (1 ml) were removed and added to glycine/HCl buffer (1 ml, 0.4 M-HCl adjusted to pH 2.75 with solid glycine). The bilirubin was then extracted with chloroform (1 m) and a sample (300 µl) was applied to a TLC plate (5 x 20 cm). The plates were developed in glacial acetic acid in chloroform (0.58% v/v). The experiment was repeated, in the absence of detergent, at pH 6.5 and with all three detergents in the pH range 5-8. In the control experiments solutions of bilirubin in cetyltrimethylammonium bromide buffer were kept in the dark for 30 min. before chromatography and assay. Merck silica gel 60 glass-backed TLC plates were activated at 120°C for 18 h and then cooled in a desiccator before use.

8.10.3 Formation of ethyl anthranilate pigments

Method modified from Blanckaert[3]. The pigments separated by thin-layer plate, were scraped off and powders transferred to centrifuge tubes containing diazotised ethyl anthranilate (1 ml). This prevented the rapid

oxidation of pigments. Formamide (2 ml) and ethanol (2 ml) were immediately added followed by vigorous mixing every 0.5 min. for 5 min. Glycine-HCl (pH 2.7, 2.5 ml) and pentan-2-one (1.5 ml) was added and shaken vigorously to extract the ethyl anthranilate azopigments. The UV absorbance of the pigment at 530 nm was recorded using a Cecil 505 Mk II spectrophotometer.

Pentan-3-one can be substituted for pentan-2-one without any change in extraction value.

In quantitative experiments, the rapid decomposition of bilirubin on dried developed chromatograms could be inhibited by spraying the chromatograms with a dilute solution (1% w/v) of ascorbic acid in water. However, the bilirubin treated in this manner could not be diazotised and estimated in the normal manner. Otherwise, a clean glass plate was placed on top of the chromatogram to prevent decomposition of bilirubin.

8.10.4 Infra-red spectroscopy of isomers

The pigments separated by TLC were scraped off into a centrifuge tube. Bilirubin was dissolved in chloroform. After centrifugation at 2,000 rpm for 1 min., the supernatant was transferred to a round bottomed flask and chloroform removed in vacuo at 50^{o}C. The pigment so obtained was ground with spectral grade KBr (1% mixture) and a wafer was formed by compression at 7.5 tons. Spectra were obtained with a Perkin Elmer 580B IR spectrophotometer.

8.10.5 Formation of isomers under aerobic and anaerobic conditions

CTAB-phosphate solution (pH 6.5) was purged for 30 min. with gaseous oxygen or nitrogen before use. A freshly prepared solution of bilirubin (2 mg, 0.33 mmol) in KOH (0.1 M, 1 ml) was added to the solution followed by irradiation with visible light.

8.10.6 Effect of free-radical inhibitions on photoisomerisation

N-tert-butyl-α-phenylnitrone (1 mg) was added to the isomerisation cocktail of bilirubin. The reducing agent, L-ascorbic acid, was also used.

8.10.7 Dehydrogenation with 2,3-dichloro-5,6-dicyano *p*-benzoquinone[30]

This mild oxidising agent dehydrogenates the pigment at the central methylene bridge to produce the fully conjugated biliverdin-IXα. The pigments separated by TLC were scraped off the plate into individual test-tubes and then dissolved in dimethyl sulphoxide. Drops of a freshly prepared solution of 2,3-dichloro-5,6-dicyano-*p*-benzoquinone in dimethyl sulphoxide (1 mg/ml) was added to the pigment until no further colour change.

8.10.8 Electron spin resonance of bilirubin

Visible light was shone on an ESR tube containing bilirubin (3 mg, 3.33 mmol) in CTAB-phosphate (10 ml, pH 6.5).

The tube was cooled to sub-zero temperature by liquid nitrogen and dry ice. The ESR of bilirubin was obtained at 30, 120 and 150 min. of irradiation.

8.10.9 Effect of irradiation of bilirubin monoglucuronide in a solution of cationic detergent

This experiment was performed by Dr. D. W. Hutchinson in the laboratory of Professor K. P. Heirwegh. Unconjugated bilirubin and bilirubin diglucuronide was removed from bilirubin monoglucuronide (2.3 A_{454} units) by TLC on silica with chloroform/methanol/water (10:5:1 v/v/v). The purified bilirubin monoglucuronide was dissolved in cetyltrimethylammonium bromide/phosphate buffer (20 µl, pH 6.5) and irradiated as described in Section 8.10.2. Glycine/HCl buffer saturated with NaCl (pH 1.8, 500 µl) was added and the solution extracted with chloroform/methanol (2:1 v/v, 500 µl). TLC of the non-aqueous extract (1.6 A_{454} units) on silica developed with chloroform/methanol/water as in Section 8.10.2 showed the presence of bilirubin diglucuronide (19%), unconjugated bilirubin (16%) and bilirubin monoglucuronide (65%), as estimated after spraying with ascorbate, with a Flying Spot TLD 100 densitometer (Vitatron, Dieren, The Netherlands).

8.11 RESULTS AND DISCUSSION

Irradiation of bilirubin-IXα in a neutral buffered solution containing a cationic detergent resulted in a rapid isomerisation to the -IIIα and -XIII isomers. The

proportion of the bilirubin-IIIα, IXα and XIIIα isomers in the equilibrium mixture was 1:2:1. Within the pH range 5-8, the maximum rate of isomerisation occurred at pH 6.5 (Fig. 8.2) with a half-life for the isomerisation reaction of 10 min.

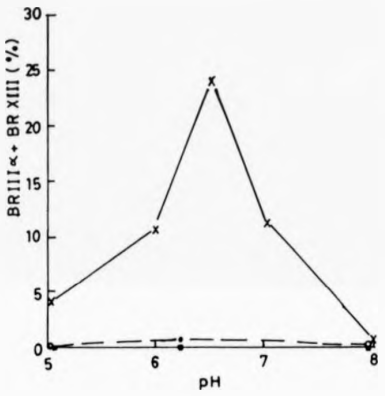


Fig. 8.2 Photoisomerisation of bilirubin-IXα in cetyltrimethylammonium bromide/phosphate buffer at different pH values.
The ordinate represents the total percentage of bilirubin-IIIα and -XIIIα after 10 min. irradiation corrected for the value of controls kept in the dark for the same period.
X = cetyltrimethylammonium bromide,
O = sodium dodecyl sulphate
□ = Tween 20

Isomerisation was undetectable in the dark after 30 min. in the absence of detergent or with neutral or anionic detergents in the pH range 5-8.

This difference in reactivity may be due to a difference in charge distribution of the detergent micelles. Presumably bilirubin is embedded in the cetyltrimethylammonium bromide micelles so that propionic acid groups are close to the positive charges on the exterior of

the micelles. The methylene bridge where bond fission must occur for isomerisation to take place is therefore in a non-aqueous environment.

This photoinduced rearrangement involves free radicals as an ESR study showed the formation of a free-radical during photoisomerisation. Also it is totally inhibited by L-ascorbic acid (1 mg/10 ml of buffer) or N-benzylidene-1,1-dimethylamine-N-oxide (1 mg/10 ml) of buffer. The latter is an efficient free radical trap[16]. Flushing the reaction with nitrogen before and during the irradiation had little effect on the rate of isomerisation since the reaction was carried out under anaerobic conditions.

The mild oxidising agent, 2,3-dichloro-5,6-dicyano-*p*-benzoquinone in dimethylsulphoxide dehydrogenated the isomers to biliverdin. This was evident from the resulting green colouration of the pigments (λ_{max} 390 nm). The bilirubin-IIIα, -IXα and XIIIα had the same polarity on TLC as the starting material. Therefore, the irradiation products did not contain appreciable quantities of the (Z,E)- or (E,E)-isomers. Their IR and UV spectra were also essentially identical to the original material. The diazopigments derived from these isomers were identical to those from the starting material.

Preliminary experiments by Dr. D. W. Hutchinson showed that bilirubin monoglucuronide disproportionates rapidly into the diglucuronide and unconjugated bilirubin when solution of the monoglucuronide in buffered cetyltrimethylammonium bromide at pH 6.5 were irradiated with visible light.

In conclusion, when bilirubin-IXα in a buffered aqueous cationic detergent at near neutral pH is irradiated with visible light it rapidly isomerised to an equilibrium mixture of the -IIIα and -XIIIα isomers. Bilirubin monoglucuronide rapidly disproportionated into unconjugated bilirubin and bilirubin diglucuronide on irradiation in a solution of the cationic detergent.

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